Determination of Catecholamines in Human Plasma by Liquid Chromatography with Electrochemical Detection

INTRODUCTION

Catecholamines are a class of biogenic amines that act as neurotransmitters. The catecholamines epinephrine, norepinephrine, and dopamine are all derived from L-tyrosine and contain a catechol (3,4-dihydroxyphenyl) nucleus and an amine group. Abberrations in catecholamine concentrations have been implicated in the etiology of depression and related disorders.[1] Catecholamines in plasma have attracted interest as markers for normal and pathological sympatho-adrenal activity in humans.[2] Plasma norepinephrine levels also provide a guide to prognosis in patients with stable, chronic, congestive heart disease.[3,4] Catecholamine levels are also used for the diagnosis and management of pheochromocytoma, a neuroendocrine tumor of the adrenal medulla.[5] This tumor is indicated by elevated catecholamine levels in plasma, typically epinephrine and norepinephrine. The correlation of abnormal catecholamine levels in various tissues to pathological conditions has emphasized a need for reliable, sensitive, and selective analytical methods for the determination of catecholamines in plasma, urine, cerebral spinal fluid, and other biological samples.

Determination of catecholamines in plasma presents the greatest challenge because catecholamine concentrations in plasma are normally in the low pg/mL range.[6] Catecholamines are also sensitive to oxidative degradation by monoamine oxidases present in circulating blood platelets. As catecholamines undergo slow oxidation in neutral and alkaline aqueous solutions, determinations in complex sample matrices are difficult. Radioenzymatic analytical methods have been used to determine catecholamines. These methods, though sensitive and specific, are laborious, require radiolabelled reagents, and cannot differentiate the three individual catecholamines without an additional thin-layer chromatography step.[6] Liquid chromatography with electrochemical detection is a direct-detection method with high sensitivity and specificity for the determination of epinephrine, norepinephrine, and dopamine in plasma. This technique also detects catechol-related substances, potentially important in both research or clinical settings where new techniques for diagnosis or drug development are needed.[7]

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Here, a sample preparation method that extracts catecholamines from plasma using a commercially available alumina extraction kit is presented. The extracted catecholamines are analyzed using the Acclaim® 120 C18 column and electrochemical detection with a disposable carbon working electrode. The run time is 30 min. The use of disposable electrodes provides reproducible analyses (electrode-to-electrode and lot-to-lot) while maintaining good peak shape, high efficiency, and excellent sensitivity. Disposable electrodes can be easily installed and also require no polishing and minimal system equilibration. Separation of major catecholamine metabolites and related substances, 5-hydroxytryptamine (serotonin), DL-normetanephrine hydrochloride, L-α-methyl-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA), and deoxyepinephrine is shown here. These compounds are reliably determined by electrochemical detection.

EQUIPMENT

Dionex ICS-3000 system consisting of:

- SP Single pump (P/N 061707), or dual pump (P/N 061713) with degas option
- DC Detector compartment (P/N 061791)
- Electrochemical detector (P/N 061719)
- Electrochemical cell (Dionex P/N 061757)
- pH-Ag/AgCl Reference electrode (P/N 061879)
- Disposable Carbon Working Electrode, Pack of 6 (with 1 mil gaskets included) (P/N 069336)
- AS Autosampler (P/N 061289) with cooling tray option (recommended)

Other Equipment

Disposable filtration units, 0.20 µm Nylon membrane (Nalgene®, 164-0020)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent for degassing eluents)

Centrifuge equipped with a ten-place, aluminum fixed-angle rotor generating a maximum RCF of 47,900 g and adapters for 15 mL conical-bottom tubes (Beckman Spinchron R, GS-6R Series, Beckman Coulter, 358702 or equivalent)

REAGENTS AND STANDARDS

ClinRep® Complete kit for catecholamines in plasma, for 250 assays (IRIS Technologies, P/N 1000)

Plasma from human (Sigma-Aldrich, P9523)

Deionized water, 18.2 MΩ-cm

Citric acid, monohydrate (Sigma-Aldrich, C1909)

Sodium acetate, anhydrous (Fluka, 71183)

Ethylenediaminetetraacetic acid (EDTA), disodium dihydrate (Sigma-Aldrich, E4884)

100 mM Octanesulfonic acid (OSA) (Dionex, 035362)

Methanol (Honeywell, 015-4)

5-Hydroxytryptamine (serotonin) (Sigma, H-7752)

DL-Normetanephrine hydrochloride (Sigma, N7127)

L-α-Methyl-DOPA (Sigma, 857416)

3,4-Dihydroxyphenylacetic acid (DOPAC) (Sigma, 850217)

3-Methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) (Sigma, H0131)

Deoxyepinephrine (Sigma, D-5886)

Norepinephrine (Aldrich, 17107-7)

Epinephrine (Sigma, E-1635)

Dopamine (BAS, 62-31-7)

3,4-Dihydroxybenzylamine (DHBA) (BAS, 16290-26-9)

Hydrochloric acid, ACS reagent, 37% (Sigma-Aldrich, 320331)

CONDITIONS

Columns	Acclaim 120, 3 µm, 120 Å, 2.1 × 150 mm Analytical (P/N 059130)
	Acclaim 120, 3 µm, 120 Å, 2.1 × 50 mm Guard (P/N 059128)
Flow Rate:	0.2 mL/min
Inj. Volume:	10 µL
Autosampler Tray Temp.:	4 °C
Detection:	DC amperometry (0.80 V, Ag/AgCl)
Data Collection Rate:	1 Hz

Method A

Eluent: 57 mM citric acid, 43 mM sodium acetate, 0.1 mM EDTA, 1.0 mM OSA, and 20% methanol

Temperature: 25 °C

Method B

Eluent: 57 mM citric acid, 43 mM sodium acetate, 0.1 mM EDTA, 1.0 mM OSA, and 10% methanol

Temperature: 30 °C

PREPARATION OF SOLUTIONS AND REAGENTS

Method A

57 mM citric acid, 43 mM sodium acetate, 0.1 mM EDTA, 1.0 mM OSA, and 20% methanol

Dissolve 11.98 g citric acid monohydrate, 3.53 g anhydrous sodium acetate, 37.2 mg EDTA, and 10 mL 100 mM OSA in 650 mL of DI water and filter through a 0.22 µm filter. Add 200 mL methanol to the filtrate and bring up the volume to 1L with filtered DI water.

Method B

57 mM citric acid, 43 mM sodium acetate, 0.1 mM OSA, and 10% methanol

Prepare as outlined in method A above, except add 100 mL methanol rather than 200 mL.

0.10 M HCl

Add 8.3 mL concentrated HCl (11.65 M) to a volumetric flask containing 500 mL DI water. Bring up the volume to 1 L with DI water.

STANDARDS

All standard concentrates can be stored (2–4 °C) for up to six months. Diluted intermediate standards are stable for three months, and working and mixed standards are stable for 2 weeks (2–4 °C).

1 mM (200 µg/mL) Standard Concentrates

Dissolve the tabulated amount of each catecholamine or related compound (Table 1) in 100 mL of 0.10 M HCl.

Table 1. Amounts for Preparation of Standard Concentrates

Catecholamine or Related Compound	Weight (mg)
DHBA	22
Dopamine	19
Epinephrine	18
Norepinephrine	21
Serotonin	39
L-Methyl-DOPA	21
3,4-Dihydroxyphenylacetic acid (DOPAC)	17
3-Methoxy-4-hydroxymandelic acid	20
Normetanephrine	22
Deoxyepinephrine	20

Intermediate 10 µM Standards

Add 1 mL of each 1 mM standard concentrate to 80 mL of 0.10 M HCl, and bring up the volume to 100 mL with 0.10 M HCl.

Working Standard Solutions

To prepare working standards, the appropriate volumes of 10 µM intermediate standards are diluted with 0.10 M HCl to desired concentrations.

Mixed Standards for Linearity Studies

For method linearity studies, epinephrine, norepinephrine, dopamine, and DHBA concentrates are diluted in 0.10 M HCl to prepare the following concentrations: 10,000, 5000, 1000, 500, 250, 100, 50, 25, 10, 5, 1, and 0.5 nM. The mixed standard solution can be stored for up to two weeks at 2–4 °C.

Standards for Interference Studies

To evaluate the separation of catecholamines from other catecholamine-related compounds, single-component standard solutions containing 100 nM of the following analytes are prepared: serotonin, DOPAC, α-methyl DOPA, 3-methoxy, 4-hydroxymandelic acid, norepinephrine, and deoxyepinephrine. Also, a mixed standard containing 50 nM epinephrine, norepinephrine, dopamine, DHBA, and 100 nM of 5-hydroxytryptamine (serotonin), DL-normetanephrine hydrochloride, L-α-methyl-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA), and deoxyepinephrine is prepared.

SAMPLE PREPARATION

Plasma Sample Preparation

The plasma extraction kit (USA Distribution by Iris Technologies) contains prepacked columns with a defined amount of activated aluminum oxide in a buffer solution. The pH of the column's buffer solution enables maximal selectivity for catecholamines. One mL of plasma was added to the activated aluminum oxide sample preparation column, followed by 50 µL of the DHBA internal standard. The aluminum oxide column was washed three times with 1 mL of the wash buffer provided in the kit to enable removal of any interfering substances that may have coabsorbed. The catecholamines were then eluted by the addition of 120 µL elution buffer (also provided with the kit). The 120 µL of eluted catecholamines were then analyzed or stored for up to two weeks at 2–4 °C.

RESULTS AND DISCUSSION

Separation

A chromatogram of a mixture of catecholamine standards using Method A is seen in Figure 1 (see the conditions section). Norepinephrine, epinephrine, DHBA, and dopamine were well-separated within 15 min, with retention times of 5.6, 6.2, 7.9, and 10.6 min, respectively. Figure 2 shows the separation of three lots of commercially available plasma with endogenous levels of catecholamines. Plasma runs were extended to 30 min in order to allow the elution of unknown peaks 5, 6, and 7.

Several catecholamine-related compounds were evaluated as single-component standards. A mixed standard was also prepared to evaluate the separation of catecholamine-related compounds from endogenous catecholamines. The best separation was achieved using the conditions described in Method B. Method B uses a lower methanol concentration and higher temperature than Method A. Figure 3 shows the separation of 50 nM norepinephrine, epinephrine, DHBA, dopamine, and 100 nM of 5-hydroxytryptamine (serotonin), DL-normetanephrine hydrochloride, L-α-methyl-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA), and deoxyepinephrine standards using Method B. There is baseline separation of all the analytes. None of the metabolites studied interfered with the determination of the catecholamines epinephrine, norepinephrine, and dopamine. Method B is recommended if the sample is expected to have large amounts of catecholamine precursors, metabolites, and drug compounds. The longer run time ensures that chromatographic interferences from previous sample injections are minimal.

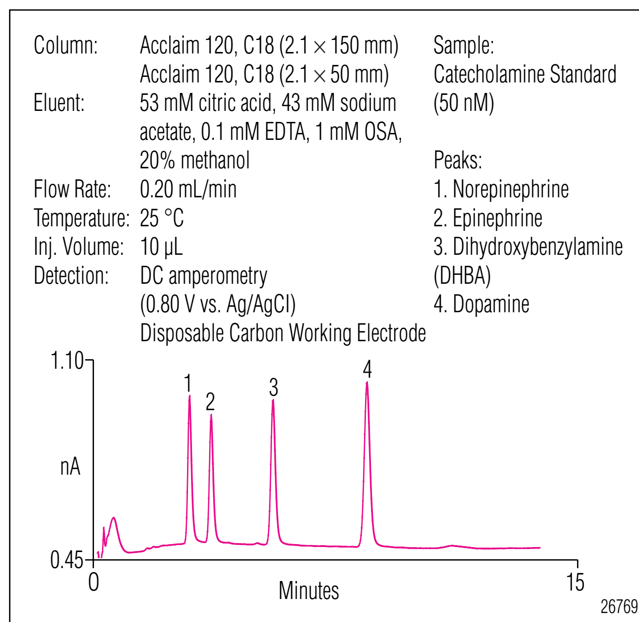

Figure 1. Separation of norepinephrine, epinephrine, DHBA, and dopamine mixed standard (Method A).

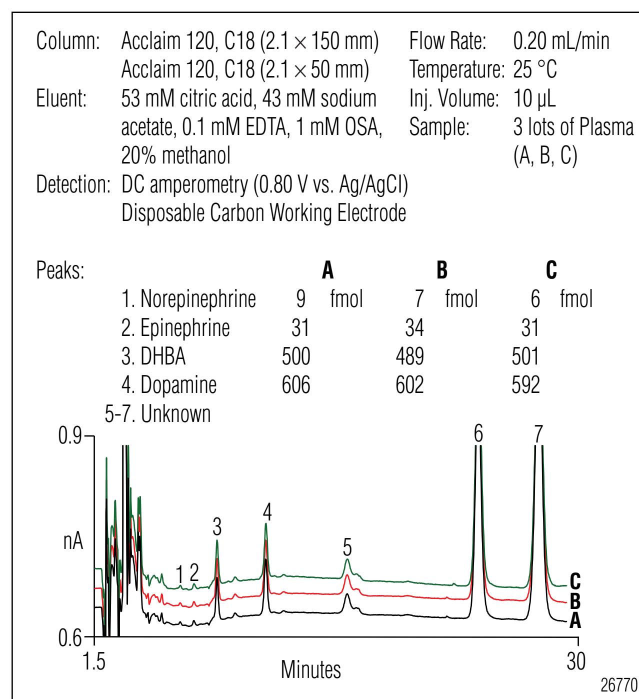

Figure 2. Separation of endogenous catecholamines and DHBA as internal standard in three different lots (A, B, C) of human plasma (Method A).

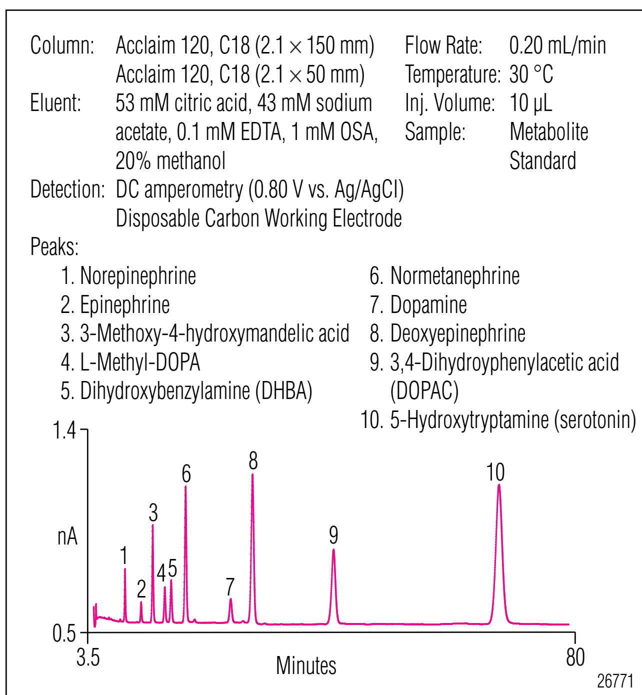

Figure 3. Separation of 50 nM norepinephrine, epinephrine, DHBA, dopamine, and 100 nM of 5-hydroxytryptamine (serotonin), DL-normetanephrine hydrochloride, L-methyl-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (vanillymandelic acid, VMA), and deoxyepinephrine standard (Method B).

Linearity

The relationship between peak area and concentration was linear over four orders of magnitude. The correlation coefficients were 0.9990, 0.9999, 0.9986, and 0.9988 for epinephrine, norepinephrine, DHBA, and dopamine, respectively.

Lower Limit of Detection and Quantitation

The U.S. Pharmacopeia (USP) defines limit of detection (LOD) as the lowest amount of analyte in a sample that can be detected but not necessarily quantified, under the stated experimental conditions.[8] In this application note, the calculation of an estimated LOD is based on the minimum concentration of an analyte which can be identified with a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ) is the minimum amount of an analyte where reliable quantification is possible, with a S/N of 10. The LOD and LOQ of epinephrine, norepinephrine, DHBA, and dopamine were calculated from the average baseline noise (peak height units) multiplied by 3 or 10, respectively, and divided by the slope obtained from the calibration curve (in peak height per concentration

Table 2. Short-Term Intraday Reproducibility of Catecholamines (50 nM)

Analyte	Conc. (nM)	RSD		
		Ret	Area	Height
Norepinephrine	50	0.14	2.4	2.8
Epinephrine	50	0.09	3.4	3.0
DHBA	50	0.10	2.3	2.2
Dopamine	50	0.09	2.0	1.9

n=30 injections

units). These estimated values were confirmed by making seven injections of a mixed standard solution fortified with catecholamines at concentrations calculated to be within approximately 3 to 5 times the S/N. The estimated LODs for norepinephrine, epinephrine, DHBA, and dopamine obtained by this method were 3.0, 4.2, 5.4, and 2.2 fmol respectively, and the LOQs were 10, 14, 18, and 7.3 fmol respectively.

Accuracy

Due to the variations in plasma from individuals, three commercially available human plasma lots were spiked with 25 nM norepinephrine, epinephrine, DHBA, and dopamine, and extracted using an alumina substrate. The mean (± SD) recoveries for norepinephrine, epinephrine, DHBA, and dopamine for the three lots were 86±6, 81±5, 95±5, and 77±4% respectively.

Precision

Short-Term Reproducibility

Table 2 shows short-term intraday reproducibility measured by injecting 30 replicates of a 50 nM catecholamine mixed standard. The intraday RSD for retention time ranged from 0.09 for dopamine to 0.14 for norepinephrine. The peak area RSD ranged from 2.0 for dopamine to 3.4 for epinephrine.

Long-Term Reproducibility

A long-term reproducibility study evaluated the performance of the method over a two-week period. Seventeen consecutive injections of standard interspersed with seven consecutive injections of plasma samples were analyzed each day. The runs were extended to 60 min to optimize sample usage, while still allowing the system to run continuously for two weeks. Under circumstances where biological fluids are present, an accumulation of reaction products on the electrode surface can result in electrode fouling.[9] The fouling of

Table 3. Concentrations of Epinephrine, Norepinephrine, and Dopamine Measured in a Plasma Lot Over a Two Week Study																	
Analyte		Measured Concentrations in Plasma (nM*)														Between-day		% Change**
		Day														Mean	RSD	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14			
Norepinephrine	Mean	50.0	48.9	49.1	49.0	48.5	48.6	48.5	48.2	48.2	48.9	49.2	47.9	47.7	47.6	48.6	1.3	-4.7
	RSD	1.0	1.0	0.5	1.1	1.0	0.3	0.3	0.3	0.3	2.4	2.6	0.4	0.2	0.2			
Epinephrine	Mean	50.1	48.4	48.7	48.5	48.3	47.9	47.6	47.3	47.3	46.8	46.7	46.8	46.5	46.4	47.7	2.2	-7.4
	RSD	1.6	1.0	0.6	0.6	0.6	0.5	0.6	0.2	0.2	0.3	0.3	0.4	0.5	0.2			
Dopamine	Mean	51.0	45.7	45.9	46.5	46.5	46.5	47.1	47.1	47.4	47.6	47.4	47.4	47.4	47.4	47.2	2.8	-7.1
	RSD	1.0	0.7	0.4	0.8	0.8	0.5	0.2	0.2	0.2	0.9	0.4	0.3	0.2	0.2			

*Concentrations corrected with DHBA internal standard
**% change in measured concentration from day 1 to day 14

the electrode can result in electrode activity loss of up to 65%. The use of an internal standard compensates for this effect. Table 3 shows the intra- and between-day variability observed for the measured concentration of catecholamines in plasma-extracted samples, corrected for the DHBA internal standard. The change in concentration from day 1 to day 14 ranged from -7.4% for epinephrine to -4.7% for norepinephrine in plasma, after correction using the internal standard.

Ruggedness

Electrode-to-Electrode Reproducibility

Four disposable carbon electrodes were tested, each over a period of three days. Seven injections were performed on each of the electrodes using a 50 nM catecholamine standard mix. These results are summarized in Table 4. The RSDs for peak area ranged from 13.9% for epinephrine to 7.7% for dopamine across the four electrodes. Disposable carbon electrodes exhibit better electrode-to-electrode reproducibility than the widely used, glassy-carbon electrodes. Electrode installation can be completed in less than 5 min, and equilibration after installation is complete in 30–60 min.

Column-to-Column Reproducibility

Table 5 summarizes retention time RSDs for 50 nM epinephrine, norepinephrine, DHBA, and dopamine evaluated on three different columns. The retention time RSDs ranged from 0.09 for norepinephrine to 0.52 for dopamine. Column 3 was manufactured using a different resin lot, and did not show any significant change in retention time compared to the other resin lot.

Table 4. Electrode-to-Electrode Reproducibility Data for Norepinephrine, Epinephrine, DHBA, and Dopamine Standards (50 nM)				
	Peak Area		Peak Height	
Analyte	Mean (nA*min)	RSD	Mean (nA)	RSD
Norepinephrine	0.027	12	0.25	12
Epinephrine	0.027	14	0.23	13
DHBA	0.032	13	0.24	12
Dopamine	0.043	8	0.25	7

n=4 electrodes, 7 injections per electrode

Table 5. Column-to-Column Reproducibility for Catecholamine Analysis				
Column #	Retention Time (min; mean±SD)			
	Norepinephrine	Epinephrine	DHBA	Dopamine
1	5.41 ± 0.01	5.99 ± 0.01	7.65 ± 0.01	10.15 ± 0.01
2	5.41 ± 0.01	5.99 ± 0.00	7.60 ± 0.01	10.05 ± 0.01
3*	5.40 ± 0.01	5.97 ± 0.00	7.59 ± 0.01	10.03 ± 0.00
Mean ± SD	5.41 ± 0.01	5.98 ± 0.01	7.61 ± 0.03	10.08 ± 0.05
RSD	0.09	0.16	0.35	0.52

*Column from a different resin lot
n=15 injections

SUMMARY

This application note method describes a rapid and sensitive method for the separation and quantitation of plasma catecholamines. The method uses an Acclaim 120, 3 µm, 120 Å column with electrochemical detection for the quantitation of fmol levels of endogenous epinephrine, norepinephrine, and dopamine within 30 min. This method uses disposable carbon electrodes that deliver high electrode-to-electrode reproducibility, and rapid electrode maintenance and equilibration upon installation.

PRECAUTIONS

Human plasma is a biohazard and should be handled as if capable of transmitting infectious agents. In the case of oral exposure, rinse the mouth with water and call a physician immediately. For inhalation exposure, move to a location with fresh air. In the case of skin contact, flush with copious amounts of water for at least 15 min. Remove contaminated clothing and shoes and then call a physician. In the case of contact with eyes, then flush with copious amounts of water for at least 15 min. Ensure adequate flushing by separating the eyelids with fingers and then call a physician. Wear protective clothing to prevent contact with skin and eyes. Store the material tightly closed at 2–8 °C. Plasma and any contaminated material should be appropriately disposed. Contact a licensed professional waste disposal organization to ensure all disposal is in accordance with existing practices employed for infectious waste at your institution. Observe all federal, state, and local environmental regulations.

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LIST OF SUPPLIERS

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Thermo Fischer Scientific (Nalgene), 75 Panorama Creek Drive, Rochester, NY 14625, Tel: 800-625-4327, www. nalgenelab.com

EMD Chemicals, (Calbiochem) Inc, 480 S. Democrat Road, Gibbstown, NJ 08027, Tel: 800-854-3417, www.emdbiosciences.com

Honeywell International, Inc, 101 Columbia Road, Morristown, NJ 077962, Tel: 973-455-2000, www.honeywell.com

Bioanalytical Systems, (BAS) Inc, 2701 Kent Avenue, West Lafayette, Indiana 47906, Tel: 765-463-1102, www.bsainc.com

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