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Note

Quantitation of amphetamine in plasma and cerebrospinal fluid by gas chromatography—mass spectrometry—selected ion monitoring, using β-methylphenethylamine as an internal standard

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Quantitative determination of amphetamine in biological samples using the selected ion monitoring (SIM) technique was reported earlier by Cho et al. [1]. They used the N-trifluoroacetyl derivative for the quantitation and d_3 (γ-C^2H_3)-amphetamine as an internal standard. The base peak at *m/e* 140 of N-trifluoroacetylamphetamine and *m/e* 143 of the internal standard were monitored for this gas chromatographic—mass spectrometric (GC—MS) quantitation. More recently, Gal [2] reported the synthesis of d_5 (ring-d_5)-amphetamine for use as an internal standard, where the fragment ions at *m/e* 91 and 96 of the N-trifluoroacetyl derivative of amphetamine and the internal standard are monitored for GC—MS analysis by electron impact ionization. Matin et al. [3] reported the use of N-pentafluorobenzoyl-S-(—)prolyl (PFBP) derivative and chemical ionization mass spectrometry to determine the d and l isomers of amphetamine.

During the last two decades street use of large quantities of amphetamines has resulted in a disproportionate number of psychotic episodes which are virtually indistinguishable from paranoid schizophrenia [4,5]. This syndrome has been termed amphetamine psychosis. We have been studying the effects of chronic administration of d-amphetamine on selected members of a primate social colony to develop an animal model psychosis [6,7]. In the course of this work on the behavioral and biochemical correlates of the effect of hallucinogens on monkey colonies, we needed to determine amphetamine levels in plasma and cerebrospinal fluid (CSF) samples in order to correlate these with

changes in the levels of dopamine metabolites in CSF. For this purpose we used the isothiocyanate derivative (NCS) of amphetamine [8,9] for the GC—MS—SIM quantitation in the electron impact mode and monitored the molecular ion at *m/e* 177. In previous work [10] we discussed the use of non-biological isomeric compounds as internal standards. In the present study we used an isomer of amphetamine, β-methylphenethylamine, as an internal standard, and compared the results with those obtained using d_5-amphetamine as another internal standard.

MATERIALS AND METHODS

d-Amphetamine sulfate, d_5-(ring) amphetamine sulfate and β-methylphenethylamine hydrochloride were used as standards. Ethyl acetate (pesticide grade) and freshly distilled carbon disulfide (AR) were used for extraction and derivatization respectively. Stock solutions of amphetamine sulfate and β-methylphenethylamine hydrochloride in water were prepared to contain 1 mg/ml each of the base. Standard solutions to contain 0.1 μg/μl of the base were prepared.

Preparation of standards

Aqueous stock solutions of amphetamine sulfate (100 μl), d_5-amphetamine sulfate (100 μl) and β-methylphenethylamine hydrochloride (100 μl) were alkalinized with 0.1 ml of 2 *N* sodium hydroxide (pH > 11) and extracted into ethyl acetate (5 ml). The ethyl acetate extracts were treated with carbon disulfide (0.5 ml), and after shaking for 15 min, they were set aside for 2 h. The organic solvent was removed under vacuum or by a current of nitrogen gas. The residue was redissolved in ethyl acetate (1 ml) to give a solution containing 0.1 μg/μl. The solutions can be stored indefinitely without any deterioration. To avoid concentration by evaporation the standards were stored in a refrigerator. Dilute solutions of the mixture of the two NCS derivatives were made from the stock solution by suitable dilution.

Standard calibration curve

To two sets of 4 tubes (duplicate) containing 2 ml each of control human plasma, 1 μg of β-phenethylamine (10 μl of standard solution) and 1 μg of d_5-amphetamine were added. To each of these tubes varying quantities 100, 200, 400 and 600 ng of standard amphetamine solution were added. The samples were made alkaline with 0.5 ml of 2 *N* sodium hydroxide, salinized with 1 g sodium chloride and extracted twice with 5 ml of ethyl acetate by shaking on a mechanical shaker for 10 min with centrifuging for 5 min at 9000 *g*. The organic extracts were pooled and reextracted into 1 ml of 0.5 *N* hydrochloric acid. After centrifuging for 10 min the organic layer was aspirated and discarded. The aqueous layer was made alkaline with 0.5 ml of 2 *N* sodium hydroxide and reextracted into ethyl acetate. As much of ethyl acetate as possible was transferred from the top layer into a dry 15-ml centrifuge tube, mixed with 0.5 ml of carbodisulfide, shaken for 15 min and set aside for 2 h. Ethyl acetate was then removed under reduced pressure, the residue redissolved in 40 μl of ethyl acetate and an aliquot of 2 μl injected into the GC—MS system.

Sample preparation

Plasma or cerebrospinal fluid. To 2 ml of plasma sample or 1 ml of CSF sample in a 50-ml glass stoppered centrifuge tube, 1 μg of β-methylphenethylamine (internal standard) was added, the sample mixed on a Vortex mixer and 0.5 ml of 2 *N* sodium hydroxide and 1 g of sodium chloride were added to the mixture. The sample was processed as described above to obtain the NCS derivative. A 2-μl aliquot of 40 μl of the final sample was used for GC—MS determination.

Red cells. Two milliliters of red cells were mixed with an equal volume of distilled water, shaken for 15 min and the internal standard (1 μg) was added and extracted in the same manner as described for the plasma sample.

Brain tissue. The tissue was homogenized in 6 volumes of 0.1 *N* methanolic hydrochloric acid, and 1 μg of the internal standard was added to the homogenate which was then centrifuged. The supernatant was separated, methanol removed under reduced pressure and the residue alkalinized with 0.5 ml of 2 *N* sodium hydroxide and extracted as described earlier.

Gas chromatography

Three different columns (180 cm long) packed with 3% SE-30 on Supelcoport, 3% OV-17 on Chromosorb W HP or 3% OV-225 on Chromosorb W HP were used for the GC studies. Carrier gas (helium) flow-rate was 30 ml/min. Column temperatures for the three columns were 170°, 180° and 190°, respectively. For routine analysis of biological samples the 3% OV-225 on Chromosorb W HP was used at 190° isothermally.

Gas chromatography—mass spectrometry

A Varian CH7 mass spectrometer interfaced with a Varian gas chromatograph 2740 through a Watson Biemann separator was used. Mass spectral conditions were: separator temperature 280°, source temperature 280° and helium flow-rate 30 ml/min with an ionizing potential of 70 eV. We have also used a Finnigan 4000 GC—MS data system for some of the assays reported here.

Animal studies

In two separate studies [6,7] individual members of a social colony of juvenile and adult stumptail macaque monkeys were administered d-amphetamine chronically. The juvenile monkeys were administered nasogastrically 0.5 mg/kg of d-amphetamine for 25 days, and the adult monkeys received 1.6 mg/kg in time release form every 12 h for 12 consecutive days. Two baseline samples of blood (heparinized tubes) and lumbar CSF samples (2.5 ml) were drawn from the experimental monkeys. Similarly, during drug treatment daily blood samples were collected in heparinized tubes at 12 noon, 3 h after the administration of the drug. Lumbar CSF samples (2.5 ml) were collected from each of the experimental adult animals on days 3, 5 and 11. We also obtained 20 control blood samples and 10 CSF samples from individual monkeys of the social colony which were free from the drug. Plasma and CSF samples were stored frozen (−40°) until they were analyzed.

We also studied the effect of d-amphetamine treatment on dopamine metab-

olites in the caudate and whole brain of the rat. Male Sprague Dawley rats (200—250 g) were administered intraperitoneally (i.p.) d-amphetamine sulfate at a dose level of 5 mg/kg. The animals were killed by decapitation at various time intervals, the brains were removed, the caudate dissected from the brain. The caudate and the rest of the brain were stored at −40° until analyzed separately for amphetamine levels.

RESULTS AND DISCUSSION

The NCS derivatives of amphetamine and β-methylphenethylamine separate on the three columns used in this study (Table I) and give a fairly abundant molecular ion (*m/e* 177) (Fig. 1), with adequate sensitivity for quantitation at the nanogram level. Fig. 2 shows (a) a SIM of d-amphetamine and the two internal standards from an aqueous extract, (b) extract of two internal standards alone from plasma and (c) extract of all three from human plasma samples. As can be seen from Fig. 2b, there was no interference from any endogenous compounds in the plasma sample. The human or monkey plasma, the rat brain tissue and monkey CSF blanks do not show any interfering peaks at *m/e* 177, the ion that is used for quantitation. In 20 samples of plasma and CSF from monkeys, the baseline of amphetamine was zero. Fig. 3a is a SIM recording of a control (drug-free) plasma extract using only one step basic extraction. Two peaks with *m/e* 177 were seen with lower retention times than the amphetamine derivative. These peaks were not seen when the two-step extraction (second acid extraction) was carried out as described under the experimental procedure. Fig. 3c is a SIM recording of an extract of a sample of plasma of a monkey treated chronically with d-amphetamine using both internal standards and one-stage extraction. Plasma and CSF levels of amphetamine in experimental monkeys in our behavioral and biochemical studies are given in Tables II and III. The monkeys received amphetamine orally in the form of time release capsules, each dose being equivalent to 1.6 mg/kg/12 h. Whole brain and caudate levels of amphetamine in rats administered 5 mg/kg of amphetamine are shown in Table IV. The level of amphetamine calculated from peak areas was 110 ng/ml with d_5-amphetamine as an internal standard and 115 ng/ml with β-methylphenethylamine as an internal standard. We have used 2 ml of plasma in five

TABLE I

GC SEPARATION OF NCS DERIVATIVES OF AMPHETAMINE AND β-METHYLPHENETHYLAMINE

RT = retention time (min); RRT = relative retention time; column length = 180 mm.

		3% SE-30 on Supelcoport 170°	3% OV-17 on Chromosorb W HP 180°	3% OV-225 on Chromosorb W HP 190°
Amphetamine	RT	1.40	1.28	1.60
	RRT	1.0	1.0	1.0
β-Methylphenethylamine	RT	1.70	1.60	1.91
	RRT	1.21	1.25	1.20

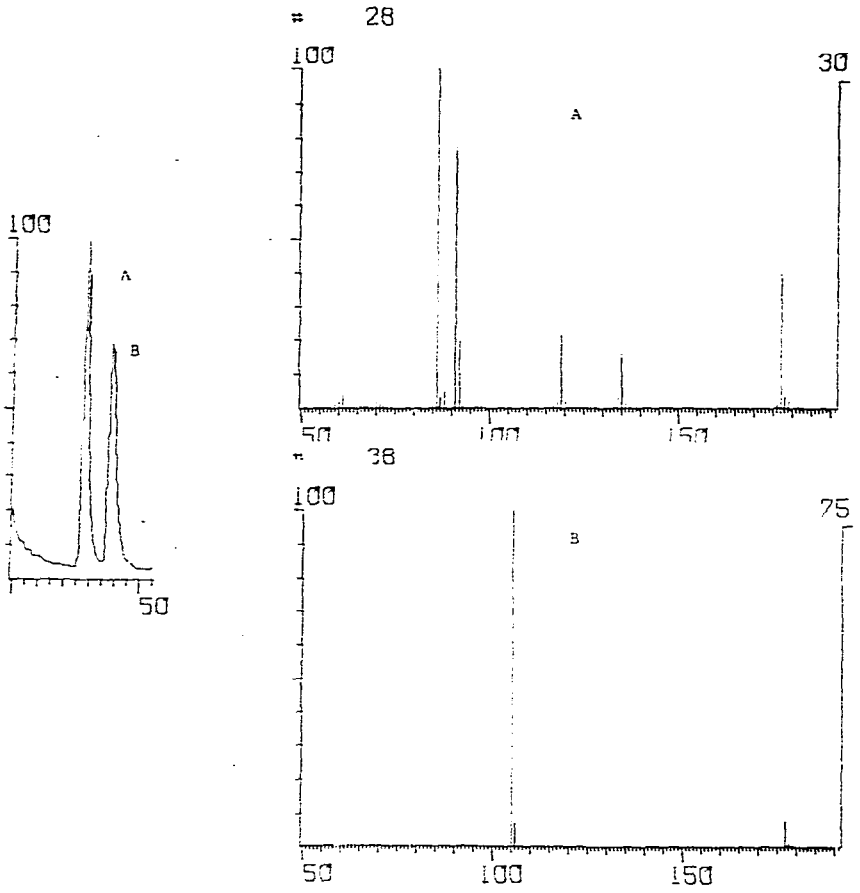

Fig. 1. GC separation of NCS derivatives of (A) amphetamine and (B) β-methylphenethylamine on 3% OV-17, and their mass spectra.

determinations where both internal standards were used. The results were within experimental error and showed a correlation coefficient of 0.95.

The isothiocyanate derivative provides a simple and elegant method for the quantitation of amphetamine in biological samples. The isomer β-methylphenethylamine, which is not of biological origin, serves as an internal standard, providing a common molecular ion for SIM, and because of its similarity in structure, serves as a carrier both in extraction procedures and on GC columns. The advantages of the NCS derivatives for the analysis of primary amines have been emphasized in earlier reports [8,9] and their specific biological application is an illustration of the general applicability of the method. Though the molecular ion *m/e* 177 is not the base peak, it provides enough sensitivity for the assay of biological samples, 1 ng in an injected sample giving a satisfactory signal-to-noise ratio of 3:1.

We have used 2 ml of plasma and 1 ml of CSF for routine analysis, and in some cases where adequate samples were not available, we have worked with 1 ml of plasma and 0.5 ml of CSF for the quantitation of amphetamine. The two-step extraction procedure and derivatization with carbon disulfide provides

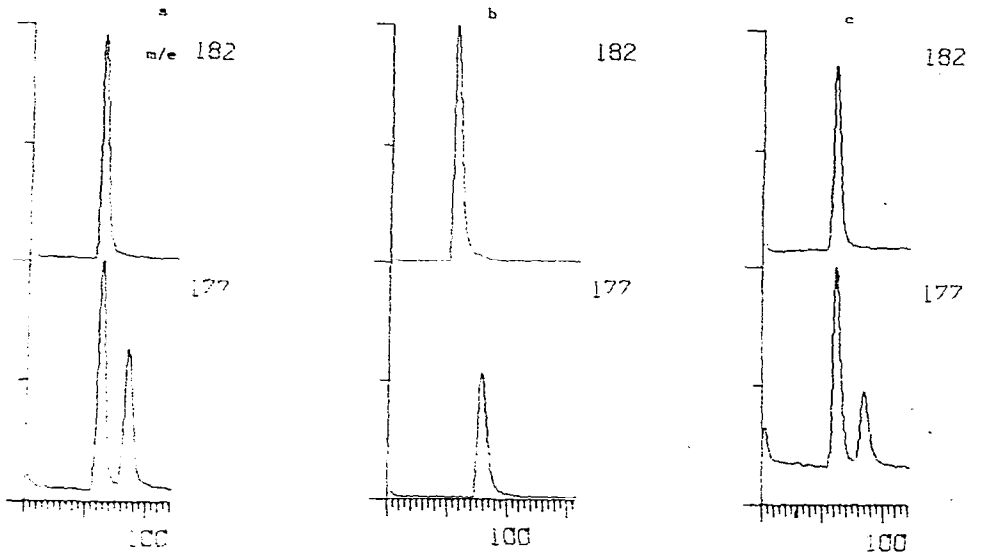

Fig. 2. (a) SIM recording of standard solution of amphetamine-NCS, β-methylphenethylamine-NCS (*m/e* 177) and d_5-amphetamine-NCS (*m/e* 182). (b) SIM recording of two internal standards added to human plasma sample. (c) SIM recording of 3 standards recovered from human plasma sample. x-Axis denotes scan number and y-axis denotes ion current intensity.

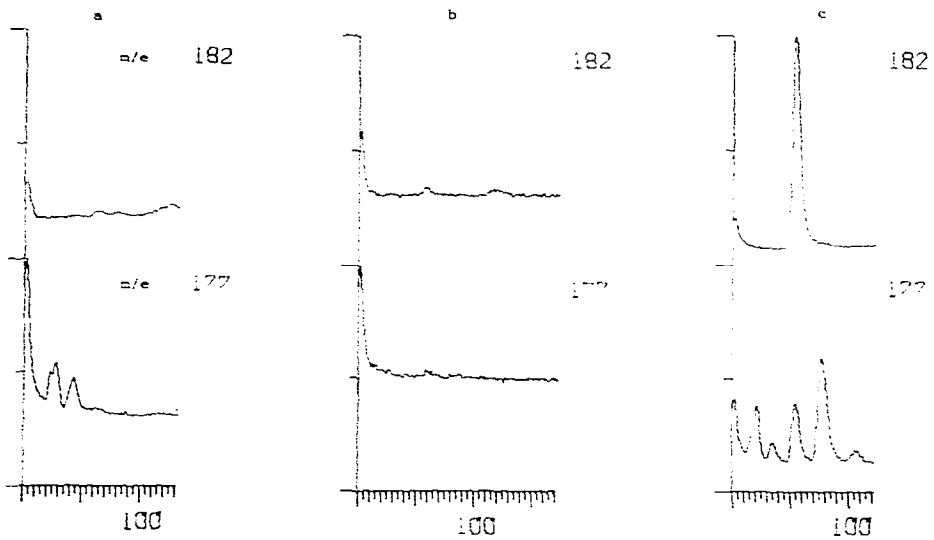

Fig. 3. SIM recording of *m/e* 177 and 182 of a control plasma sample (a) after one-step extraction; (b) after two-step extraction and (c) amphetamine plasma samples with two internal standards after one-step extraction. Details are given in the text. x-Axis denotes scan number and y-axis denotes ion current intensity.

a clean-up procedure, and no interference is noticed at *m/e* 177 or *m/e* 91 when used for increased sensitivity. The NCS derivative is superior to the dansyl derivative which is less volatile and pentafluoropropyl or N-trifluoroacetyl derivatives which do not give the molecular ion in the electron impact mode.

TABLE II

LEVELS OF AMPHETAMINE (ng/ml) IN PLASMA SAMPLES OF MONKEYS TREATED WITH d-AMPHETAMINE

Monkeys 1 and 2 (adult) were administered time release capsules nasogastrically 1.6 mg/kg every 12 h for 12 days. Samples were drawn at 12 noon 3 h after administration. Monkeys 3 and 4 (juvenile) were administered 0.5 mg/kg/day nasogastrically for 25 days.

No. of days after administration	Monkey 1	Monkey 2	Monkey 3	Monkey 4
0 (baseline)	0	0	0	0
1	50	77.5	40	72
2	120	166	90	72
3	108	100	n.d.*	n.d.
4	115	134	n.d.	n.d.
5	130	70	n.d.	n.d.
7	n.d.	n.d.	122	90
11	110	127	n.d.	n.d.
15			45	31
21			132	60
25			100	50

*n.d. = not done.

TABLE III

LEVELS OF AMPHETAMINE (ng/ml) IN CEREBROSPINAL FLUID SAMPLES OF MONKEYS TREATED WITH d-AMPHETAMINE

Values expressed as mean ± S.D. of two determinations. Monkeys were administered time release capsules at a dose level of 1.6 mg/kg/12 h.

Day after	Monkey 1	Monkey 2
3	83.1 ± 0.5	106.0 ± 1.0
5	93.0 ± 0.6	69.1 ± 1.8
11	100.6 ± 0.6	80.8 ± 0.7

TABLE IV

LEVELS OF AMPHETAMINE IN THE CAUDATE AND WHOLE BRAIN LESS CAUDATE OF RATS AFTER ADMINISTRATION OF d-AMPHETAMINE (5 mg/kg i.p.)

Values expressed as mean ± S.D. of two determinations. Number of animals = 3.

Min after administration	Caudate (μg/g)	Whole brain less caudate (μg/g)
30	2.25 ± 0.70	4.1 ± 1.5
120	0.42 ± 0.15	0.7 ± 0.3

Unlike the acyl derivatives which are not stable, the NCS derivatives could be stored indefinitely without loss due to decomposition.

We have stored a mixture of standards containing 20 ng/μl each of amphetamine-NCS and β-methylphenethylamine-NCS for over a year and compared it

with freshly prepared standards once a month and did not find any measurable decomposition. Further, the quantitative response in the GC-MS system was relatively constant for both isomers. Thus in 20 determinations of the same mixture the ratio of peak heights or peak areas of the two components in the mixture varied less than 5%, well within limits of experimental error in such biological determinations. The results from both internal standards agreed within ± 5%.

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