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SELECTED ION MONITORING ASSAY FOR BIOGENIC AMINE METABOLITES AND PROBENECID IN HUMAN LUMBAR CEREBROSPINAL FLUID

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SUMMARY

Details are presented of an improved selected ion monitoring assay for the major biogenic amine metabolites and probenecid in human lumbar cerebrospinal fluid (CSF). The metabolites and probenecid are simultaneously extracted with ethyl acetate from an acidified aqueous phase, and are simultaneously converted to pentafluoropropionyl esters by reaction with pentafluoropropionic anhydride and pentafluoropropanol. The esters of the metabolites are analyzed following a single injection of the derivatized sample onto the gas chromatographic column, while the ester of probenecid is analyzed following a separate injection onto the gas chromatographic column. Quantitation is achieved using for internal standards deuterated analogues of the metabolites and a chemical analogue of probenecid. Data are presented on the concentration of free and conjugated forms of the metabolites in lumbar CSF taken from healthy volunteers.

INTRODUCTION

A primary goal of recent studies on affective disorders in humans has been to compare the concentrations of neurotransmitters and neurotransmitter metabolites in the body fluids of normal and affected subjects. In particular, the concentrations of 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), the major central nervous system metabolites of serotonin, dopamine, and norepinephrine, respectively, have been measured in urine and lumbar cerebrospinal fluid (CSF) in an attempt to describe the neurochemical abnormalities which may occur during mania, depression, and schizophrenia [1, 2]. In this report we describe details of an integrated selective ion monitoring (SIM) method for measuring the concentrations of 5-HIAA, HVA,

MHPG, 3,4-dihydroxyphenylacetic acid (DOPAC), and *p*-(di-*n*-propylsulfamyl)-benzoic acid (probenecid), in small volumes of human lumbar CSF. This methodology is of note because by simultaneously extracting and derivatizing these compounds the quantification is achieved with the same aliquot of CSF, thus providing a valuable economy of time and materials. We also report, for the first time, the concentration of free and conjugated forms of the metabolites in human lumbar CSF taken from healthy volunteers.

METHODS

Reagents

Pentafluoropropionic anhydride (PFPA) was obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.); 2,2,3,3,3-pentafluoro-1-propanol was from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.); HVA, MHPG (piperazine salt), and DOPAC were from Calbiochem (Los Angeles, Calif., U.S.A.); 5-HIAA and 5-methoxyindole-3-acetic acid (5-CH_3OIAA) were from Regis Chemical Co. (Chicago, Ill., U.S.A.); sulfatase (arylsulfatase containing some β-glucuronidase, type H-1) was from Sigma; probenecid was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); 5-hydroxyindole-3-acetic-2,2-d_2 acid (d_2-5-HIAA, 98 atom % ^{2}H) ^2H_2O (99.7 atom % ^{2}H), $C^2H_3COO^2H$ (99.5 atom % ^{2}H) and ^{2}HCl (99 atom % ^{2}H) were from Merck (Rahway, N.J., U.S.A.); borane methylsulphide complex was from Aldrich; MHPG sulfate was kindly donated by Hoffman La Roche (Nutley, N.J., U.S.A.) (Ro 4-6028); 3% (w/w) OV-17, 80—100 mesh Gas-Chrom Q, was from Applied Science Labs (State College, Pa., U.S.A.). All other reagents and compounds were of the highest purity available.

Collection of CSF

The subjects were normal males, none of whom had received psychotropic drugs for at least three weeks prior to the study. Having given informed consent, the subjects received two lumbar punctures in the lateral decubitus position, after an overnight fast, before arising from bed. The lumbar punctures were performed at 8.00 a.m. on consecutive days, and prior to the second lumbar puncture the subjects were administered probenecid in six oral doses of 12.5 mg/kg and a final oral dose of 25 mg/kg for a total of 100 mg/kg, given 18, 16, 14, 12, 10, 8 and 3 h before the lumbar puncture. A total of 24 ml of CSF was collected in 6-ml aliquots, from each lumbar puncture, and the second 6-ml aliquot was used for the analyses described here. No CSF specimens were used which contained blood; immediately after collection ascorbic acid was added (1 μmole/ml) and the samples were frozen and stored at —70° until they were analyzed.

Preparation of deuterated internal standards

Deuterated internal standards were prepared by exchange reactions [3, 4] in ^2H_2O—^{2}HCl. Assessment of the isotopic composition and purity of the products was made by combined gas chromatography—mass spectrometry (GC—MS) of the pentafluoropropionyl (PFP) esters (prepared as described below) after a correction was made for the natural isotope satellite ion in the authentic non-deuterated compound. In the case of vanillylmandelic acid (VMA) and MHPG

it was not possible to weigh the deuterated products accurately. In this situation SIM analysis, using the non-deuterated compound as a standard, was employed to determine the concentration of d_3-VMA and d_3-MHPG in solution.

DOPAC. A solution of HVA (300 mg) in ^2H_2O (2 ml) and ^2HCl (2 ml, 38% in ^2H_2O) was heated in a sealed glass tube at 130° for 16 h. After cooling, the mixture was extracted three times with 5 ml of ethyl acetate. The pooled organic phase was shaken with activated charcoal, filtered, and dried under vacuum (< 40°). On standing, the resulting oil crystallized. Recrystallization from an ether—benzene mixture yielded needles which were collected by filtration. The final product contained d_5-DOPAC with less than 1% d_0-DOPAC, and no detectable amount of HVA.

HVA. A solution of HVA (30.5 mg) in ^2H_2O (2 ml), ^2HCl (2 ml, 38% in ^2H_2O) and $C^2H_3COO^2H$ (0.5 ml), was gently refluxed in an open-ended test-tube on a heating block at 190° for 30 min. After cooling, the mixture was extracted three times with 5 ml of ethyl acetate, and the cooled organic phase was taken to dryness under vacuum (< 40°). The resulting oil crystallized on standing, and recrystallization from ether–benzene yielded needles containing d_3-HVA with less than 1% d_0-HVA.

VMA. A solution of VMA (400 mg) in ^2H_2O (6 ml), ^2HCl (10 ml, 20% in ^2H_2O) and $C^2H_3COO^2H$ (2.5 ml) was heated gently under reflux in an open-ended test-tube (15 cm × 2.5 cm) on a heating block at 170° for 15 min. The mixture was then quickly cooled on ice, filtered under vacuum, and extracted three times with 3 ml of ethyl acetate. The pooled organic phase was shaken with activated charcoal and filtered again. The yellow-red filtrate was dried under vacuum (< 40°), and the resulting oil was dissolved in H_2O (1 ml) and loaded onto a Sephadex G-10 column (11.9 × 1.0 cm) suspended in H_2O (Pharmacia, Uppsala, Sweden). As the column was eluted with H_2O, 5-ml fractions were collected. Aliquots (100 μl) of each fraction were removed, dried under a stream of nitrogen, and derivatized (as described below) for examination by GC—MS. Fractions 2, 3, and 4 contained VMA and were pooled and lyophilized to dryness, yielding a yellow powder (weighing approximately 10 mg) which contained d_3-VMA with less than 3% d_0-VMA.

MHPG. To a solution of d_3-VMA (5 mg of the powder containing d_3-VMA) in tetrahydrofuran (1 ml) was added borane methyl sulphide complex (1 ml, containing approximately 5% methyl sulphide). The mixture was left to stand at room temperature with occasional manual agitation for 1 h, after which anhydrous methanol (2.5 ml) was added slowly. After drying under a stream of nitrogen, the resulting clear oil was dissolved in ^2H_2O (2.5 ml). The final solution contained d_3-MHPG with less than 1% d_0-MHPG and a trace of d_3-VMA but no detectable amount of d_0-VMA.

Synthesis of m-(di-isobutylsulfamyl)benzoic acid (DBSB)

DBSB was synthesized from *m*-(chlorosulfonyl)benzoic acid and di-isobutylamine as already described [5].

Sample preparation for unconjugated monoamine metabolites and probenecid

To a 1.0-ml aliquot of CSF are added 100 μl of an aqueous solution containing d_5-DOPAC (10 pmoles), d_3-HVA (400 pmoles), d_3-MHPG (15

pmoles), d_2-5-HIAA (400 pmoles), 5-CH_3OIAA (500 pmoles) and ascorbic acid (100 nmoles). A solution of DBSB (44 nmoles) in 20 μl of 20 m*M* NaOH is then added, and the sample, which is kept on ice (0—4°) during the solvent extraction process, is acidified by the addition of formic acid (4 *N*, 50 μl) and extracted twice with freshly redistilled ethyl acetate (4 ml each time) using centrifugation to separate the phases (3000 *g* for 5 min). The organic phases are pooled in a screw-capped test-tube and taken to dryness under a stream of nitrogen. The dried contents are washed to the bottom of the tube by addition of anhydrous methanol (100 μl), which is evaporated under a stream of nitrogen. The drying process is completed by addition of benzene (50 μl), which is also evaporated under a stream of nitrogen.

Hydrolysis of conjugated forms of the monoamine metabolites

To a 1.0-ml aliquot of CSF containing the internal standards, aryl sulfatase (type H-1, 100 units in 100 μl of 1 *M* sodium acetate buffer. pH 6.2) is added. The solution is incubated on a gently shaking water-bath at 37° for 1 h, after which it is extracted in the same manner as described for the free monoamine metabolites.

Preparation of standard curves

With each set of samples (free and hydrolyzed), a standard curve is prepared for each compound by addition of the internal standards and known amounts of each compound to samples of artificial CSF (containing m*M* concentrations of NaCl (140), KCl (3.4), $CaCl_2$ (1.3), $MgCl_2 \cdot 7H_2O$ (0.54), urea (0.22), NaH_2PO_4 (0.25), Na_2HPO_4 (0.25), glucose (3.33), and $NaHCO_3$ (3.57). These standard samples are then treated in the manner already described for the free and conjugated metabolites.

Derivatization of monoamine metabolites and probenecid [6]

Pentafluoropropionic anhydride (40 μl) and 2,2,3,3,3-pentafluoropropanol (10 μl) are added to the dried ethyl acetate extracts, and the mixture is heated to 75° for 15 min. The tubes are then cooled and dried under a stream of nitrogen. Additional pentafluoropropionic anhydride (50 μl) is added, and the mixture heated to 75° for a further 5 min. Excess reagent is again removed in a stream of nitrogen, and the dried residue is stored at —70° until analyzed.

Gas chromatography

The derivatives of the monoamine metabolites are separated on a silanized glass column (5 ft. × 2 mm I.D.) packed with 3% OV-17 (80—100 mesh) with helium as the carrier gas (25 ml/min) and the injector port at 200°. The column temperature is 115° and is increased at a rate of 6°/min from the time of sample injection. The derivatives of probenecid and DBSB are chromatographed on the same column but with the injector port at 250° and the column isothermal at 200°.

Typically, for analysis of the monoamine metabolites the derivatized sample, prepared from an extract of 1 ml of lumbar CSF, is dissolved in 20 μl of ethyl acetate of which 2—5 μl are injected onto the GC column. For analysis of probenecid and DBSB the remainder of the derivatized extract is dissolved in a

further 100 μl of ethyl acetate of which 1—2 μl are injected onto the GC column.

The gas chromatograph was provided with a toggle switch to divert the solvent from the mass spectrometer; the diverter was switched off 0.5 min after sample injection.

Mass spectrometry

Mass spectrometry was carried out with a Finnigan 3200 GC—MS system integrated with a Finnigan 6000 data system. The parameters of the source are manipulated to maximize the signal at *m/e* 464 (obtained from standard calibration gas: perfluoro-tri-*n*-butylamine). The system was provided with a baffle; ethanol was used as the cooling agent and a cryocool unit (Neslabs Instruments) afforded the refrigeration.

RESULTS

Monoamine metabolites

Under the conditions used, the PFP derivatives of DOPAC, VMA, MHPG, HVA, 5-HIAA, and 5-CH_3OIAA separated from one another on the OV-17 column (Table I). The mass spectra of these derivatives (Table I) are dominated by the presence of relatively intense molecular ions with characteristic losses of 177 and 163 mass units due respectively to losses of the esterified carboxyl function ($OCOCH_2C_2F_5$) and an esterified hydroxyl function ($OCOC_2F_5$). Other characteristic fragments correspond to the loss of 147 and 150 mass units, due presumably to loss of C_2F_5CO and $C_2F_5CH_2OH$, respectively, from the molecular ions.

Using the technique of multiple-ion SIM, we have been able to identify DOPAC, MHPG, HVA, and 5-HIAA in extracts of human CSF (Table II). There is also a small peak in the *m/e* 445 ion trace at the retention time of VMA, but we have been unable to confirm the identity of VMA with peaks in other ion traces characteristic of this compound.

The parameters used for quantitative SIM data collection of the PFP derivatives of the monoamine metabolites can be chosen so that DOPAC, VMA, MHPG, HVA and 5-HIAA are analyzed during the same GC run (Table III). In this case, the ion at *m/e* 445 is used for quantitative measurements of both d_0-VMA and d_0-MHPG, and the ion at *m/e* 448 can be used for quantitative measurements of both d_3-VMA and d_3-MHPG. Otherwise, the close proximity of the DOPAC, VMA, and MHPG peaks would not permit the analysis of all compounds in the same GC run. We have not routinely measured VMA concentrations in human CSF samples, however, because the amount present is usually below the sensitivity limit of our mass spectrometer. Therefore, in the 2.5—3.3-min time frame, we monitor the ions at *m/e* 458 and 622 (d_0-MHPG), and *m/e* 461 and 625 (d_3-MHPG), and thereby obtain a stronger signal for the MHPG peak. In this case, the ratio of peak heights in the ion traces at *m/e* 458 and 461 is used in the quantitative measurements.

The degree of completion of hydrolysis of conjugated forms of the metabolites was checked with time-course experiments using 230-μl aliquots of artificial CSF samples obtained from rat brain perfusion experiments [7].

GAS CHROMATOGRAPHIC RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF THE PFP DERIVATIVES OF THE MONOAMINE METABOLITES

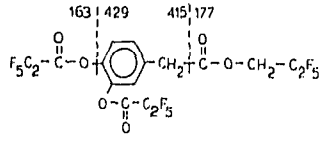
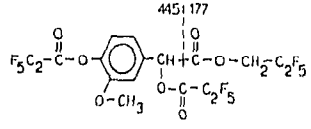
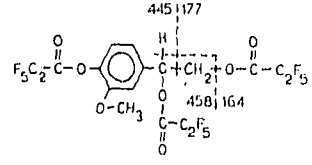
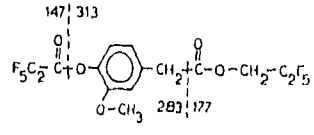
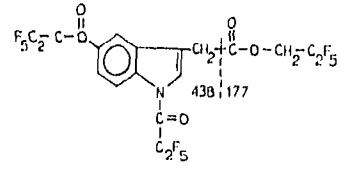
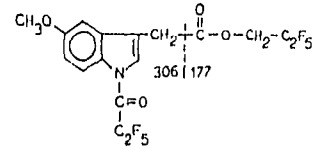
Compound	Structure of derivative and probable fragmentation pattern	Retention time (min)*	Major mass spectral ions (*m/e*) and their relative intensities (%)
DOPAC		2.35	592 (44%, M^+), 429 (19%, $M^+ - 163$), 415 (100%, $M^+ - 177$), 387 (28%)
VMA		2.63	622 (33%, M^+), 472 (28%, $M^+ - 150$), 445 (100%, $M^+ - 177$), 417 (28%)
MHPG		3.04	622 (43%, M^+), 458 (100%, $M^+ - 164$), 445 (52%, $M^+ - 177$), 417 (26%)
HVA		3.90	460 (100%, M^+), 313 (13%, $M^+ - 147$), 283 (6%, $M^+ - 177$)
5-HIAA		6.37	615 (30%, M^+), 438 (100%, $M^+ - 177$)
5-CH_3OIAA		9.21	483 (100%, M^+), 306 (88%, $M^+ - 177$)

TABLE II

SIM IDENTIFICATION OF THE DERIVATIVES OF DOPAC, MHPG, HVA, and 5-HIAA IN EXTRACTS OF HUMAN CSF

Compound	Source	Retention time (min)	Ions monitored (*m/e*) and ratio of peak intensities (%)
DOPAC	Authentic standard	2.34	592 (35%), 415 (100%), 387 (40%)
	Hydrolyzed CSF	2.17	592 (57%), 415 (100%), 387 (35%)
MHPG	Authentic standard	3.04	622 (41%), 458 (100%), 445 (53%), 417 (25%)
	Unhydrolyzed CSF	3.00	622 (51%), 458 (100%), 445 (58%), 417 (27%)
	Hydrolyzed CSF	2.90	622 (51%), 458 (100%), 445 (59%), 417 (27%)
HVA	Authentic standard	3.90	460 (100%), 313 (25%), 283 (29%)
	Unhydrolyzed CSF	3.93	460 (100%), 313 (26%), 283 (31%)
	Hydrolyzed CSF	3.82	460 (100%), 313 (27%), 283 (33%)
5-HIAA	Authentic standard	6.37	615 (27%), 438 (100%)
	Unhydrolyzed CSF	6.29	615 (27%), 438 (100%)

TABLE III

PARAMETERS USED DURING QUANTITATIVE SIM DATA COLLECTION FOR DOPAC, VMA, MHPG, HVA, AND 5-HIAA

Time after sample injection (min)	Ions monitored (*m/e*)	Compound	Ratio of peak heights used in the quantitation
1.5—2.5	415, 592	DOPAC	592/597
	420, 597	d_5-DOPAC	
2.5—3.3	445, 622	VMA	445/448
	448, 625	d_3-VMA	
	445, 622	MHPG	445/448
	448, 625	d_3-MHPG	
3.3—5.3	460	HVA	460/463
	463	d_3-HVA	
5.3—7.3	438, 615	5-HIAA	438/440
	440, 617	d_2-5-HIAA	

Using the Sigma H-1 enzyme preparation (which is a mixture of aryl sulfatase and β-glucuronidase) we found that the concentration of the free forms of DOPAC, MHPG, and HVA in the samples increased during the first hour of the incubation and thereafter remained constant.

Deuterated internal standards which were carried through the extraction and derivatization procedure were found to contain the same distribution of deuterium as the standards which had been converted directly to the PFP derivatives. This result indicates no loss of deuterium occurred by back-exchange with hydrogen during the extraction process.

Complete disappearance of DOPAC was found to occur when solutions of artificial CSF (250 μl) containing DOPAC (24 and 48 pmoles) were incubated for 2 h at 37° prior to extraction and derivatization. No detectable amount of breakdown of DOPAC occurred, however, when ascorbic acid was added to the CSF (1 μmole/ml of CSF) and the solutions were kept at 0° for 2 h.

The recovery of 5-HIAA (500 pmoles) during ethyl acetate extraction from solutions of artificial CSF (1.0 ml) in the absence of added 5-CH_3OIAA, was found to be 5%, but the recovery was increased to 37% by the addition of 100 μg of 5-CH_3OIAA to the artificial CSF. Use of ethyl acetate that had not been freshly redistilled immediately before the extractions resulted in variations between replicate samples in the intensity of the signals obtained for DOPAC and 5-HIAA. This variation was avoided by redistilling the ethyl acetate immediately before use. Variability between replicate samples in the intensity of the peaks for DOPAC, MHPG, HVA and 5-HIAA was also obtained when no care was taken to dry the ethyl acetate extracts completely prior to derivatization. This source of variability was overcome when, prior to derivatization, the residue remaining after the ethyl acetate was blown off in the nitrogen stream was first washed to the bottom of the tubes with methanol then dried in a nitrogen stream, and then dried again in a stream of nitrogen after addition of benzene. Using optimized conditions, the recovery of the monoamine metabolites during extraction was checked by adding DOPAC (540 pmoles), VMA (40 pmoles), MHPG (220 pmoles), HVA (440 pmoles), and 5-HIAA (500 pmoles) to 1.0 ml of artificial CSF. Percentage recoveries, calculated by

comparison with the peak intensities obtained from the same amount of compound which was derivatized directly, were 31%, 75%, 79%, 80%, and 40% for DOPAC, VMA, MHPG, HVA, and 5-HIAA, respectively.

In the concentration ranges for DOPAC, VMA, MHPG, HVA, and 5-HIAA of 2—200, 1—100, 5—140, 6—1400, and 5—1300 pmoles, respectively, the standard curves are linear. Inverse linear regression analysis has been used to determine the concentrations of DOPAC, MHPG, HVA, 5-HIAA, and probenecid in human lumber CSF taken before and after probenecid administration (Table IV).

Probenecid

Details of the procedure used in the SIM analysis of probenecid have been presented elsewhere [5], so only a brief synopsis will be given here. The PFP derivatives of probenecid and DBSB elute from the GC column with similar retention times (Table V). The mass spectra of these derivatives are characterized by weak molecular ions (< 1%) with base peaks at *m/e* 388 and 402 for probenecid and DBSB, respectively, corresponding to losses of C_2H_5 and C_3H_7 from the molecular ions (Table V). The quantitative assay is based on recording, in the SIM mode, the peaks in the *m/e* 388 and 402 ion traces. In the concentration range of 4—100 nmoles, the standard curve is linear, and inverse regression analysis has been used to determine the concentration of probenecid in lumbar CSF of patients given an oral load of 351 nmoles/kg of the drug (Table IV).

DISCUSSION

We have described a sensitive SIM assay for the quantitation of DOPAC, VMA, MHPG, HVA, 5-HIAA, and probenecid in 1.0-ml aliquots of human lumbar CSF. The method, which employs a chemical analogue as an internal standard for probenecid and deuterated analogues as internal standards for all other compounds, utilizes the fact that the chemical properties of DOPAC, VMA, MHPG, HVA, 5-HIAA, and probenecid permit their simultaneous extraction and derivatization in adequate yield for SIM analysis.

The mass spectral data presented (Table II) verify the occurrence of MHPG, HVA, and 5-HIAA in human lumbar CSF. The absence of significant amounts of other compounds co-eluting with these compounds validates the SIM procedures used for their quantitation in extracts of human lumbar CSF. In derivatized extracts of hydrolyzed and unhydrolyzed human CSF DOPAC eluted in close proximity to a compound which contributed a significant signal to the *m/e* 415 and 387 ion traces. This made it difficult to measure accurately the intensity of the DOPAC peak in these two ion traces and accounts for the differences obtained (Table II) in the relative intensities of the peaks in the 592, 415, and 387 ion traces between authentic DOPAC and DOPAC in human CSF samples. The *m/e* 592 (d_0-DOPAC) and 597 (d_5-DOPAC) ion traces, however, were not confused with extraneous peaks and the intensities of the peaks in these ion traces have been used in the quantitative measurements.

Sensitive and reliable SIM assays for measuring the concentrations of several catecholamine metabolites but no serotonin metabolites in the same sample of CSF, using different extraction procedures and different

TABLE IV

MEAN CONCENTRATIONS (± S.E.) OF COMPOUNDS IN HUMAN LUMBAR CSF BEFORE AND AFTER PROBENECID ADMINISTRATION

Subjects were 23 normal males: age range 22—69 years; mean age 43 years; mode 20—30-year age bracket.

Compound	Form of metabolite			Conjugated (percentage total)
	Free	Total	Conjugated	
Before probenecid administration				
DOPAC (pmoles/ml)	2.38 ± 0.22 (21)*	3.80 ± 0.31 (18)	1.41 ± 0.54 (17)***b	37
MHPG (pmoles/ml)	48.40 ± 2.70 (23)	53.10 ± 3.30 (22)	2.40 ± 0.80 (22)***c	5
HVA (pmoles/ml)	239.50 ± 20.10 (23)	241.10 ± 20.20 (22)	1.40 ± 3.20 (22)	0.6
5-HIAA (pmoles/ml)	153.90 ± 13.40 (23)	Not done		
After probencid administration**				
DOPAC (pmoles/ml)	21.00 ± 4.41 (10)	22.00 ± 2.85 (12)	0.60 ± 0.84 (9)	2.7
MHPG (pmoles/ml)	59.51 ± 4.46 (13)	62.83 ± 4.57 (13)	3.32 ± 1.47 (13)***a	5.3
HVA (pmoles/ml)	957.64 ± 75.44 (14)	961.04 ± 75.93 (14)	3.00 ± 11.10 (44)	0.3
5-HIAA (pmoles/ml)	608.95 ± 53.66 (14)	Not done		
Probenecid** (nmoles/ml)	47.80 ± 5.4 (12)	Not done		

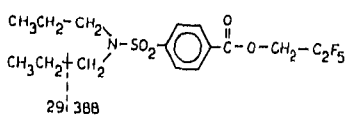
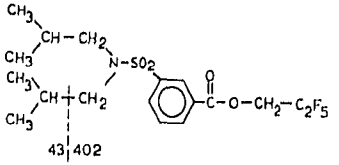
*Figure in parentheses refers to the number of subjects whose CSF concentrations were used in the calculation of each mean and standard error.

**Subjects were administered probenecid at 100 mg/kg (351 μmoles/kg) in six divided oral doses of 12.5 mg/kg and a final oral dose of 25 mg/kg, taken 18, 16, 14, 13, 10, 8 and 3 h, respectively, prior to the lumbar puncture.

***Significantly greater than zero using a one-tailed Student's *t*-test. [a]$p < 0.025$; [b]$p < 0.01$; [c]$p < 0.005$.

TABLE V

GAS CHROMATOGRAPHIC RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF THE PFP DERIVATIVES OF PROBENECID AND DBSB

Compound	Structure of derivative and probable fragmentation pattern	Retention time (min)*	Major mass spectral ions (*m/e*) and their relative intensities (%)
Probenecid		1.89	417 (<1%, M^+), 388 (100%, $M^+ - 29$)
DBSB		2.00	445 (<1%, M^+), 402 (100%, $M^+ - 43$)

*Column: 3% OV-17 isothermal at 210°.

derivatives than those used in this report, have been previously described 6 by Karoum and others [4, 8, 9]. Swahn et al. [10] have described a SIM method for the simultaneous extraction and derivatization of the catecholamine metabolites MHPG and HVA and the serotonin metabolite 5-HIAA from human CSF. We have improved the sensitivity of this method for 5-HIAA and have extended the assay to include the additional catecholamine metabolites DOPAC and VMA and the drug probenecid. By adopting some relatively simple procedural changes we have also been able to improve the reliability of the assay for DOPAC, MHPG, HVA and 5-HIAA.

In order to obtain high recoveries of DOPAC it was found essential to maintain an ascorbic acid concentration of 1—2 m*M* in the aqueous phase during the extraction process. Also, to avoid large losses of DOPAC, we found it necessary to reduce the time of hydrolysis of the conjugated metabolite. In contrast to described procedures [4, 10—12], 1 h at 37° with the aryl sulfatase—β-glucuronidase enzyme preparation proved to be sufficient to hydrolyze completely the conjugates in samples of rat brain perfusate. The concentrations of conjugated forms of DOPAC, MHPG, and HVA in rat brain perfusion fluid is much greater than the concentration of these compounds in human CSF [7]. Therefore, if the conjugates in human CSF are hydrolyzed with equal efficiency with the H-1 enzyme preparation, 1 h at 37° is sufficient to complete hydrolysis of conjugated forms of these compounds in human lumbar CSF.

In quantitating the conjugated metabolites, the approach we have taken has been to measure free and total metabolite concentrations in separate aliquots of CSF, and thus to determine the concentration of the conjugated metabolite by difference. An alternative approach used by others [4, 11, 12] has been to measure conjugated metabolite concentrations directly in samples of CSF. This has been done by first removing the free metabolites by extraction, and then hydrolyzing the remaining conjugated metabolite. This approach suffers from the disadvantage of measuring either artifactually high conjugated metabolite concentrations, if the free metabolite is not completely removed by extraction, or artifactually low conjugated metabolite concentrations, if a significant proportion of the conjugated metabolite is removed during extraction.

Bertilsson et al. [13] found that the addition of 11 nmoles of 4-hydroxyindole-3-acetic acid to a 2-ml aliquot of CSF improved the recovery of 5-HIAA. In the work reported here the recovery of 5-HIAA was found to be significantly improved by the addition of 500 pmoles of 5-CH_3OIAA to the samples prior to extraction, and by using freshly redistilled ethyl acetate for the extractions. The use of 5-CH_3OIAA benefits from the advantage that the PFP derivative elutes after the 5-HIAA derivative and thus does not confound measurement of the 5-HIAA peak intensity.

In addition to their satisfactory GC and mass spectral characteristics and ease of preparation, the PFP derivatives of the metabolites and probenecid were all found to be relatively stable. When stored dry, they are stable for at least two weeks at −70°.

Takahashi et al. [9] have previously reported the concentrations of VMA, HVA and isohomovanillic acid in human lumbar CSF taken from healthy controls, but neither the concentrations of DOPAC, MHPG, and 5-HIAA in

lumbar CSF taken from healthy control subjects before probenecid administration, nor the concentration of DOPAC, MHPG, HVA, 5-HIAA and probenecid in lumbar CSF from healthy control subjects taken after probenecid administration have been previously reported. We report concentrations of the free forms of DOPAC, MHPG, HVA, 5-HIAA and probenecid in lumbar CSF taken from healthy control subjects, before and after probenecid administration, which are within the concentration ranges reported for these compounds in lumbar CSF taken from non-psychotic hospitalized patients [4, 10, 11, 13]. We have also demonstrated the presence of small but significant amounts of conjugated forms of DOPAC and MHPG in lumbar CSF, which is consistent with previous reports [11, 12, 14, 15].

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