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SINGLE-STEP METHOD FOR DERIVATIZATION OF DOPAMINE AND SOME RELATED COMPOUNDS IN AQUEOUS MEDIA FOR GAS CHROMATOGRAPHY

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SUMMARY

The present report describes a single-step method for derivatization of dopamine and several structurally related compounds, either a catecholic or a monophenolic amine, acid, alcohol or glycol present in aqueous solutions. Also, nanogram levels of these compounds may be assayed by gas chromatography with electron-capture detection following derivatization. For the determination of optimum reaction conditions, aqueous solutions of either [^{14}C]dopamine or [^{3}H]norepinephrine were reacted with the derivatization agent, heptafluorobutyryl chloride. A mass spectrum of the derivative of dopamine confirmed the formation of triheptafluorobutyryl dopamine. To determine the sensitivity and specificity of the derivatization method, a number of biological samples from rats and humans were analyzed for dopamine and 3,4-dihydroxyphenylacetic acid. The urinary analyses showed that conjugation may be the major metabolic pathway for dopamine and 3,4-dihydroxyphenylacetic acid in rats as well as in humans. The present method should prove convenient to determine the urinary sulfate conjugate of 3,4-dihydroxyphenylacetic acid, a non-invasive indicator of central nervous system dopaminergic activity, and other catecholamine metabolites of clinical interest.

INTRODUCTION

Chemical determinations of dopamine (DA) and related compounds when present in biological samples are difficult because of their low concentrations. Also, the sensitivity of any catecholic compound to oxidative degradation and the presence of amphoteric functional groups make isolation of these compounds from aqueous biological samples troublesome.

Potential utility of derivatization of amines in aqueous media has been demonstrated by Brooks and Horning [1]. Thus, anhydrates [2,3] and alkyl chloroformates [4,5] have been employed to react with amines and phenols in aqueous media. Also, the stable acetyl or carbamate derivatives may be extracted into a relatively non-polar solvent. For gas chromatography (GC), however, these

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methods often require two steps: the initial one followed by a solvent extraction and further derivatization under anhydrous conditions. This report describes a reagent that may acylate both phenolic and amine groups in a single step in an aqueous medium for extraction with a non-polar solvent and for GC. Furthermore, resulting derivatives are suitable for quantitation by electron-capture detection (ECD). Currently, high-performance liquid chromatographic (HPLC) techniques for the determination of DA and related compounds at low levels have become popular. GC–ECD, however, also provides a high sensitivity. Additionally, definitive mass spectrometric (MS) confirmation may be easily achieved. GC–MS analysis of these derivatives also permits the use of safe stable isotope-labelled compounds in clinical metabolic studies. The application of this reagent to derivatize a number of DA-related compounds and to quantify DA and its important metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) present in biological samples is also presented in this report.

EXPERIMENTAL

Chemicals and reagents

[2-^{14}C]DA (specific activity 8–10 mCi/m*M*) and [7-^{3}H]norepinephrine (NE) (10 800 mCi/m*M*) were purchased from New England Nuclear (Boston, MA, U.S.A.) and the labelled NE was mixed with the cold amine. Sigma (St. Louis, MO, U.S.A.) supplied DA, N-methyl-DA, L-DOPA, NE, *p*-tyramine (TA) and 3-methoxytyramine (MTA) as hydrochlorides, epinephrine bitartrate (EPI), amphetamine sulfate and homovanillic acid (HVA). From Aldrich (Milwaukee, WI, U.S.A.), 3,4-dihydroxyphenylpropionic acid (DPPA) was purchased. Calbiochem supplied 2-amino-6,7-dihydroxy-1,2,3,4-tetra-hydronapthalene hydrobromide (ADTN), DOPAC, 3-methoxy–4-hydroxyphenethylglycol piperazine salt (MHPG), and 3,4-dihydroxynorephedrine (α-methylnorepinephrine, MNE). Heptafluorobutyryl chloride (HFBCl) was obtained from Alpha Products (Danvers, MA, U.S.A.) and 3 *M* hydrochloric acid in *n*-butanol from Regis Chemicals (Morton Grove, IL, U.S.A.). Fisher Scientific (Springfield, NJ, U.S.A.) supplied methyl *tert.*-butyl ether and *n*-hexane. These solvents were further purified by distillation over lithium aluminum hydride. Aluminum oxide (Fisher Scientific) was boiled in 6 *M* hydrochloric acid, thoroughly washed with water and oven-dried. The phosphate buffer was 1.0 *M* dipotassium hydrogenphosphate at pH 7.5.

Samples

Sprague–Dawley rats (200–250 g, female) were decapitated using a guillotine. The brain and adrenal glands were immediately chilled in ice-cold saline and within 3–5 min the brain areas were dissected. Weighed tissue samples were then quickly homogenized in ice-cold 1 *M* hydrochloric acid and the extracts were saved after centrifugation. Rat urine samples were collected for 12-h periods over 2 *M* hydrochloric acid in metabolic cages. Sucrose (100 ml, 10%) was the source of calories per day for 24 h before and during the collection period to avoid dietary

interference [6]. Human urine was collected over 2 *M* hydrochloric acid for the reported periods. Acid hydrolysis of conjugated DA and DOPAC was achieved in 1 *M* sulfuric acid at 100°C [7]. The rats were given intraperitoneally either saline (no drug treatment), tetrabenazine methane sulfonate (Hoffman-La Roche, Nutley, NJ, U.S.A.), reserpine (Serpasil, Ciba, Summit, NJ, U.S.A.), amphetamine sulfate or cocaine hydrochloride before brain and adrenal analysis. L-DOPA was administred before urine collection.

Derivatization

Solutions of either standard compounds (2.5 ng/μl in 0.01 *M* hydrochloric acid) or of those extracted from biological samples, as described below, were dried under nitrogen at 55°C. These samples were then redissolved in 200 μl of pH 7.5 phosphate buffer. A 5-μl volume of HFBCl reagent was added to the solution with intermittent vortexing for 5 min or otherwise specified periods. The derivative was then extracted into 2.0 ml *n*-hexane which was washed twice with water (0.2 ml). The organic fraction was evaporated to dryness. The extracted derivative was redissolved in a suitable volume of *n*-hexane and an aliquot (1.0–5.0 μl) was injected for GC. The carboxylic acids, DOPAC and HVA, were first esterified by heating at 100°C for 30 min with 10–25 μl of 3 *M* hydrochloric acid in *n*-butanol before their reaction with HFBCl.

For the derivatization of [^{14}C]DA and [^{3}H]NE, dried aliquots of their solutions (230 ng of DA and 25 ng of NE) were redissolved in 1.0 ml phosphate buffer and then reacted with varied volumes (0–50 μl) of HFBCl for 0–30 min as specified in Results. The derivatives were then extracted, after potassium chloride saturation, with 2.0 ml ethyl acetate which was assayed for radioactivity in a Beckman LS-100C liquid scintillation counter.

For determination of DA and DOPAC in biological samples, the initial step for purification and concentration, after adding the respective internal standards ADTN and DPPA, was the absorption on alumina at pH 8.4–8.6. Following a thorough washing of alumina with water, DA and DOPAC were eluted with 2.0 ml 1 *M* hydrochloric acid. DOPAC was extracted from the eluate with methyl *tert.*-butyl ether. After removal of the solvent, it was esterified and reacted with HFBCl as described above. A suitable aliquot of the aqueous eluate was dried under nitrogen for the derivatization of DA as described for the standard compounds.

Instrumentation

The analyses were carried out in a Perkin-Elmer Model 3920 gas chromatograph with a ^{63}Ni electron-capture detector. The column was 1.83 m$\times$2 mm I.D. packed with SP-2250 (Supelco, PA, U.S.A.) and the flow-rate of the gas mixture argon–methane (95:5) was 20 ml/min. The oven temperature was 150°C or as noted in Results with the injector and the detector set at 325 and 300°C, respectively. All of the quantitations were based upon the corresponding calibration curves of nanogram versus peak-height ratios using the internal standards. Standard mixtures of DA with ADTN and of DOPAC with DPPA were run through the analytical steps as described above for constructing the calibration curves.

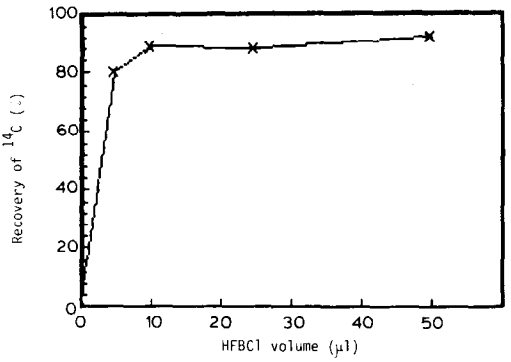

Fig. 1. Derivatization of [^{14}C]DA with different reagent volumes.

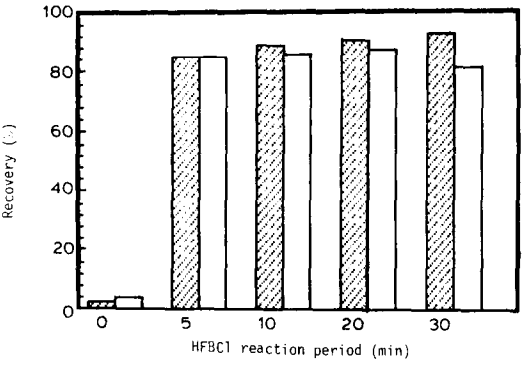

Fig. 2. Derivatization of [^{14}C]DA (▨) and [^{3}H]NE (□) at varied reaction periods.

RESULTS

HFBCl volume and reaction period

The percentages derivatization of the labelled catecholamines at varied HFBCl volumes and reaction periods are indicated by Figs. 1 and 2, respectively. Fig. 1 shows that 5 μl of HFBCl (reaction time 5 min) in 1.0 ml buffer yielded about 80% recovery of [^{14}C]DA into the organic solvent. The corresponding figure was about 90% after larger volume of either 10, 25 or 50 μl of reagent. Generally, either 5 μl reagent reacted in 200 μl phosphate buffer or 25 μl reagent in 1.0 ml buffer lead to equally complete (90%) derivatization. As expected, ommission of HFBCl reagent led to no radioactivity in the organic solvent (Fig. 1). Fig. 2 indicates that the derivatization of either [^{14}C]DA or [^{3}H]NE reached the maximum by 5 min and organic extractable radioactivity remained the same up to 30 min reaction time. For practical considerations, 5 min were taken as optimum reaction period.

Reference compounds

Table I shows the retention times and peak heights of DA, NE and EPI. The table also includes their various metabolites and other related compounds. The

TABLE I

RETENTION TIME AND RELATIVE PEAK HEIGHTS OF REFERENCE COMPOUNDS

Compound	Characteristic groups	Retention time at 150° (min)	Relative peak height (1.0 ng)
DA	OH, OH, NH_2	3.0	100
ADTN	OH, OH, NH_2	8.0	40
N-methyl DA	OH, OH, $NHCH_3$	3.0	84
DOPAC	OH, OH, (COOH)	2.5*	91
DPPA	OH, OH, (COOH)	4.0*	48
HVA	OH, (OCH_3), (COOH)	4.0**	33
MTA	OH, (OCH_3), NH_2	6.0	60
TA	OH, NH_2	2.0	150
NE	OH, OH, CHOH, NH_2	2.0	57
MNE	OH, OH, CHOH, ($CHCH_3$), NH_2	1.5	75
MHPG	OH, (OCH_3), CHOH, CH_2OH	1.5	89
EPI	OH, OH, CHOH, $NHCH_3$	2.0	43

*At 160°C.
**At 170°C.

characteristic group present in these compounds derivatized with HFBCl in aqueous media was one or more of the following: a primary or secondary amine, a phenol, a catechol, an alcohol, or a glycol. It was also observed that the derivative of DA could be detected to as low as 25 pg (approximately five times the noise) and only the TA derivative provided a greater sensitivity under the chromatographic conditions. These derivatives were found to be stable for the same-day analysis and some of these maintained their peak heights 24 h later.

Mass spectrometry

For a confirmation of the formation of triheptafluorobutyryl derivative of DA, an electron-impact mass spectrum was obtained (LKB 9000) which showed a small peak at *m/e* 741, the molecular ion, an intense peak at *m/e* 528 ($M-NH_2COC_3F_7$), other relatively intense peaks at *m/e* 331 and 226 and a bare peak at *m/e* 169. All these ions are characteristic of the expected perfluoro derivative of DA after reaction with HFBCl. Furthermore, a negative-ion chemical ionization spectrum (Hewlett-Packard 5985B) indicated a high sensitivity as may be expected from such a derivative; this spectrum showed a peak at *m/e* 721 (741 − H − F).

Analysis of biological samples

A number of biological samples from rat and human sources have been analyzed for DA and DOPAC derivatized with HFBCl, following initial purification (see *Samples*). Table II shows the levels of DA in rat striatum, brain stem, and adrenal glands and that of DOPAC in striatal tissue. In saline-treated rats, a high (10.8 ng/mg tissue) level of DA was present in the striatum which also contained 1.22 ng/mg DOPAC. Tetrabenazine at 30 min markedly reduced the DA level in striatum with a large increase in DOPAC content. Reserpine at 24 h reduced DA

TABLE II

RAT TISSUE LEVELS OF DA AND DOPAC

The results are averages of three to seven determinations with the standard error of the mean. For the purification and derivatization steps see *Derivatization*.

Treatment	DA content (ng/mg of tissue)			DOPAC content (ng/mg of tissue)
	Striatum	Brainstem	Adrenal	Striatum
None	10.80 ± 0.78	0.10	7.01 ± 0.57	1.22 ± 0.11
Tetrabenazine (1 h, 5.0 mg/kg)	2.06 ± 0.44*	0.05	3.75 ± 0.17*	3.60 ± 0.45*
Reserpine (24 h, 2 × 2.5 mg/kg)	1.10 ± 0.10*	–	10.63 ± 0.64*	1.76 ± 0.16*
Amphetamine (0.5 h, 5.0 mg/kg)	12.34 ± 0.30	–	4.54 ± 0.36*	0.59 ± 0.05*
Cocaine (0.5 h, 25.0 mg/kg)	12.18 ± 0.53	–	6.51 ± 0.54	1.26 ± 0.05

*$p < 0.05$ versus no treatment.

content by about 90% and elevated the DOPAC level, and amphetamine clearly reduced the DOPAC concentration. DA in brain stem was about 1% of that in striatum and was further reduced by tetrabenazine. Fig. 3 represents a typical chromatogram from brain tissue DA determinations.

Although adrenal glands are known to contain high levels of EPI and NE, DA

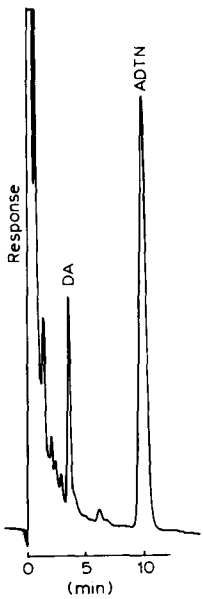

Fig. 3. Gas chromatogram of DA from brain tissue extract. GC column, 10% OV-17 at 170 °C.

TABLE III

FREE AND CONJUGATED DA AND DOPAC IN RAT AND HUMAN URINE

The results are averages of three to five determinations with the standard error of the mean.

Urine source	DA			DOPAC		
	Free (μg)	Conj. (μg)	Conj./ total (%)	Free (μg)	Conj. (μg)	Conj./ total (%)
Rat (12 h) (no treatment)	939.2 ± 197.1*	189.1 ± 52.5*	17.2 ± 4.7	2719.8 ± 359.6*	3391.9 ± 148.3*	56.1 ± 2.5
Rat (12 h) (L-DOPA)**	1958.7 ± 199.4	589.1 ± 289.6	22.4 ± 10.1	–	–	–
Human (6h)	101.1 ± 11.0	176.6 ± 51.7	61.2 ± 7.4	357.9 ± 102.7	206.5 ± 46.3	37.1 ± 3.0
Human (24 h)	293.4 ± 24.6	696.9 ± 200.3	67.7 ± 7.0	1202.9 ± 220.0	861.5 ± 220.1	40.9 ± 2.8

*ng.

**100 mg/kg, two times at 0 and 6 h.

was also found at 7.01 ng/mg level in rats without any drug treatment. Adrenal DA was also affected by the drug treatments and it was elevated by reserpine while tetrabenazine and amphetamine reduced it.

Peyrin and co-workers [6,8] have suggested that the urinary conjugated DOPAC level may reflect central nervous system (CNS) dopaminergic activity, and the present methods utilizing GC of HFBCl derivatives have been applied to determine free and conjugated DOPAC and DA in rat and human urine (Table III). Rat urine contained DA, and a significant fraction (17.2%) of it was in conjugated form. Treatment of the rats with 100 mg/kg L-DOPA (two times) lead to massive increases of free (2085-fold) and total (2258-fold) DA; the conjugated fraction was slightly elevated (22.4%). Human urine also contained free and conjugated DA but percent conjugated were much higher in both 6-h (61.2%) and 24-h (67.7%) samples. These results show that rat (average body weight 0.22 kg) urine contained 356.9 and 71.9 ng/kg body weight per h of free and conjugated DA, respectively. The corresponding human (average body weight 61 kg) values were 276.0 and 482.1 at 6 h and 199.5 and 473.9 at 24 h, respectively.

DOPAC was present also in free and conjugated forms in rat and human urine. Conjugated DOPAC was 56.1% in rat and the corresponding human figure was 37.1% at 6 h and 40.9% at 24 h. The rat values were 1033.5 and 1288.9 ng/kg/h for free and conjugated DOPAC. The human subjects produced 977.1 and 563.7 ng/kg/h of free and conjugated DOPAC at 6 h and 818.0 and 585.8 ng/kg/h at 24 h. It also appears from these results that excretion rates of free and conjugated DOPAC in human urine at 6 h did not differ markedly from those at 24 h; free and conjugated DA excretion rates were also similar at those two periods.

DISCUSSION

Results in Figs. 1 and 2 indicate that the derivatization of DA and NE may occur within 5 min at room temperature with 5–25 μl of HFBCl reagent. Also,

these derivatives could be conveniently extracted with a low-polarity solvent as *n*-hexane and routinely washed with water leading to cleaner chromatograms from biological samples. Unspent HFBCl is hydrolyzed in the reaction medium to produce free–salt forms of the corresponding acid and these forms are lost during water wash and volatilization at low temperatures (50–55°C). Generally, aliphatic acid chlorides are rapidly, almost explosively, hydrolyzed in water [9] but HFBCl appears to be sufficiently stable in the phosphate buffer medium to derivatize nanogram levels of DA and the other compounds (Table I).

The results in Table I indicate that not only DA but also NE, EPI and a number of the biologically important metabolites of catecholamines may be conveniently derivatized in aqueous medium by HFBCl. The sensitivity of an electron-capture detector to these highly fluorinated derivatives provides a routine method for the determinations of nanogram levels of these compounds by GC. For specificity, it may be combined with MS. The results in Table I also indicate that some compounds bearing a characteristic group other than those in DA may also yield derivatives suitable for GC. Among these, alcohols and glycols do react with acid chlorides [9].

Because of our interest in the dopaminergic actions of tetrabenazine, reserpine, amphetamine and cocaine [10,11], the present derivatization method was tested to quantify striatal DA and DOPAC levels (Table II) after administration of these drugs. The data are consistent with the known DA-releasing actions of tetrabenazine and reserpine and are in conformity with these drug actions observed by others [12,13]; the observed increase of DOPAC level after these two drugs are also as expected. As reported before [14], amphetamine only slightly increased (Table II) the DA level, and the decrease of DOPAC after amphetamine, as observed, has been well documented [15].

DA in adrenal glands, unlike that in striatum, has been little investigated, primarily because of the difficulty of quantitating its level in the presence of a large (100-fold) excess of other catecholamines [16]. The present method clearly separates the derivative of DA from those of NE and EPI (Table I). The observed adrenal (average weight 46 mg/pair of adrenal per 220 g rat body weight) DA level (Table II) is close to the reported value [16].

A number of authors [17–19] have employed various methods to determine body fluid levels of conjugated DA for experimental and clinical purposes. The present results (Table III) indicate that urinary conjugated DA may be conveniently quantified by GC of the HFBCl derivative. It appears, however, that conjugation in human, mostly sulfation [20], was much greater than that in rats (Table III) and may reflect the high level of circulating conjugated DA in humans [21]. Use of glucoronidase-free sulfatase for hydrolysis should add further specificity to the determination of the sulfate form.

A major part of CNS DOPAC appears to be conjugated prior to elimination [7] and the results of Peyrin et al. [8] have suggested that a determination of urinary conjugated DOPAC may provide a non-invasive method to examine CNS dopaminergic activity. Various techniques employing radioenzymatic methods, fluorimetry, colorimetry and HPLC with several prepurification steps [6,17,22,23] have been used to determine DOPAC and DA in rat and human urine. The pres-

ent derivatization method in conjunction with a simple prepurification step using alumina provide a high sensitivity in routine GC–ECD determinations for urinary DOPAC and DA. Complete reliability may be provided by additional MS, either by electron-impact mass fragmentography or by the negative-ion chemical ionization method.

It is also apparent from our results (Table I) that a number of other clinically important metabolites of DA and NE, including HVA and MHPG, may be derivatized by HFBCl in aqueous medium thus providing GC–ECD methods for their routine determinations.

Since a large number of endogenous biological compounds bear reactive groups as those present in DA, NE and their metabolites, HFBCl may prove to be useful in many other determinations as well.

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