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Methadone radioimmunoassay: two simple methods

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Two simple and economical radioimmunoassays for methadone in blood or urine are described. Haemolysis, decomposition, common anticoagulants and sodium fluoride do not affect the results. One assay uses commercially-available [1-^{3}H](−)-methadone hydrobromide as the label, while the other uses a radioiodinated conjugate of 4-dimethylamino-2,2-diphenylpentanoic acid and L-tyrosine methyl ester. A commercially-available antiserum is used in both assays. Normethadone and α-methadol cross-react to a small extent with the antiserum while methadone metabolites, dextropropoxyphene, dipipanone and phenadoxone have negligible cross-reactivities. The 'cut-offs' of the two assays as described are 30 and 33 ng ml^{-1} for blood, and 24 and 21 ng ml^{-1} for urine. The assay using the radioiodinated conjugate can be made more sensitive if required by increasing the specific activity of the label.

Methadone is used as an antitussive, as an analgesic and in maintenance therapy for opiate addicts. Blood concentrations vary from about 50 ng ml^{-1} to over 1 μg ml^{-1} depending on the size of the dose and the frequency of administration, while considerably higher amounts corresponding to 5% or more of the dose are excreted unchanged in a 24 h urine sample (Inturrisi & Verebely 1972a, b). Methadone is encountered often enough in forensic toxicology to warrant the development of specific 'screening' methods to identify positive samples. Radioimmunoassay (RIA) is a particularly useful technique for this purpose since it is sensitive, economical and well-suited for handling samples in batches. This paper describes two RIAs for methadone in blood or urine. Haemolysis, decomposition, common anticoagulants and sodium fluoride do not affect the results.

Various immunoassays for methadone have been developed. The methadone Emit-dau enzyme immunoassay (Syva) and a haemagglutination inhibition method (American Drug Research Institute, PO Box 134, Park Forest, Il. 60466, U.S.A.) based on the method of Liu & Adler (1973) are both commercially available. They are, however, intended for urine analysis and are unsuitable for assaying the small, haemolysed blood samples often submitted for forensic analysis. A methadone Abuscreen RIA (Roche Diagnostics) has been described (Manning et al 1976) but is not marketed. Bartos et al (1977) developed an RIA for methadone using [^{3}H](±)-methadone and an antiserum from rabbits immunized with a (±)-methadol-hemisuccinate-thyroglobulin conjugate. McGilliard et al (1979) used similar methods to produce stereospecific RIAs for (+)- and (−)-methadone. Ling et al (1981) described a methadone RIA using [^{3}H](±)-methadone and an antiserum from Hoffmann-La Roche Inc.

We have developed two RIAs, A and B, for methadone. [^{3}H](−)-Methadone is used as the label in Assay A while a radioiodinated methadone derivative is used in Assay B. The antiserum from an Emit-dau methadone kit is used in both assays.

MATERIALS AND METHODS

Buffer

0·067 M phosphate of pH 7·4 containing 0·2% bovine γ-globulin (Cohn Fraction II, Sigma Chemical Co., Fancy Road, Poole, Dorset, U.K.) and 0·1% sodium azide.

Antiserum

Antiserum from an Emit-dau methadone kit (Syva UK, Syntex House, St. Ives Road, Maidenhead, Berkshire, U.K.) is stored at 2 °C and diluted when required with buffer to bind 50–60% of the label in the absence of unlabelled methadone. The dilutions are determined from antiserum dilution curves and are approximately 1:30 for Assay A and 1:50 for assay B.

Methadone standards

(±)-Methadone in buffer is stored in silanized glass vials at 2 °C. Suitable ranges are 0–250 ng ml^{-1} for Assay A and 0–100 ng ml^{-1} for Assay B.

* Correspondence.

PEG solution
Polyethylene glycol (PEG, mol. wt 6000) 23% w/v in buffer containing no γ-globulin.

Scintillant
2,5-Diphenyloxazole (PPO), 4 g, and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), 0·2 g, litre^{-1} reagent-grade toluene.

Sample preparation. Assay A
Urine is assayed directly. Blood (75 μl) is buffered to pH 9 and extracted twice with 2 ml aliquots of diethyl ether (extraction efficiency 100%). The combined extracts are evaporated to dryness, taken up in 150 μl of buffer and assayed. Blood extracts or urine are diluted further with buffer if necessary.

Sample preparation. Assay B
Blood or urine (50 μl) is diluted with an equal volume of buffer. This is done to conserve the sample. Further dilution may be necessary to ensure the result lies within the range of the standard curve.

[1-^{3}H](−)-*Methadone hydrobromide*
This was purchased from New England Nuclear (2 New Road, Southampton, U.K.). It was supplied in ethanol and had a specific activity of 4·7 GBq mmol^{-1}. It is stored at 2 °C and diluted to 1·5 ng ml^{-1} for use in the assay.

Reaction of (±)-4-dimethylamino-2,2-di-phenyl-pentanoic acid (DDA) and (L)-tyrosine methyl ester (TME) to give isomeric DDA-TME conjugates
DDA, 50 mg (American Drug Research Institute), TME, 33 mg, and dicyclohexylcarbodiimide, 70 μl, were dissolved in dry dimethylformamide, 10 ml, and left for several days at 2 °C. Dicyclohexylurea was removed by filtration then the solution was reduced to small volume on a rotary evaporator and the products were separated by high-performance liquid chromatography (h.p.l.c.) on a 25 × 1 cm column of 5 μm silica baked at 800 °C. The eluant was methanol–water–conc. ammonium hydroxide–70% nitric acid (900:89:9:2) adjusted to pH 8·0 at a flow-rate of 4 ml min^{-1}. A 25 μl injection loop and a variable-wavelength u.v. detector set at 260 nm were used. Preliminary experiments showed that one peak in the chromatogram (Fig. 1A) contained material that cross-reacted with the antiserum (using Assay A) and could be radioiodinated. Some of the material was isolated by collecting fractions from 20 h.p.l.c.

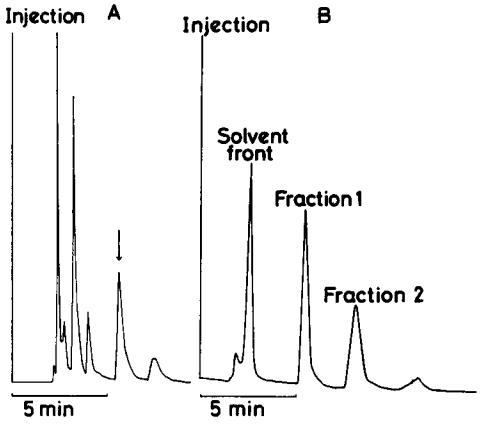

FIG. 1. H.p.l.c. purification of isomeric DDA-TME conjugates. See text for conditions. A. H.p.l.c. of reaction mixture. Material from the arrowed peak was isolated by preparative h.p.l.c. B. H.p.l.c. of material from the arrowed peak in A. Fractions 1 and 2 were isolated by preparative h.p.l.c.

runs. The pooled fractions were evaporated to dryness and taken up in methanol. Further analysis showed that the product consisted of two main components, Fractions I and II, which were isolated by h.p.l.c. under the same conditions as before, but with the eluant adjusted to pH 10 (Fig. 1B). The isolated fractions, each weighing approximately 500 μg, were evaporated to dryness, taken up in 0·5 ml methanol and stored at −20 °C. Fractions I and II both cross-reacted with the antiserum and could be radioiodinated. We concluded that they were isomers resulting from the reaction of (±)-DDA with L-TME (Fig. 2). This was confirmed by

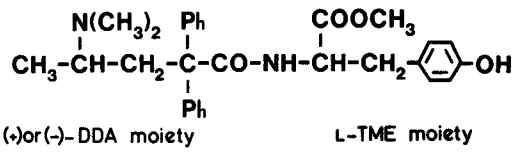

FIG. 2. Structure of isomeric DDA-TME conjugates.

mass spectrometry in the electron impact mode when the fractions gave similar spectra containing characteristic ions of methadone (*m/z* 72, 165, 193, 265) and a molecular ion of the expected *m/z* 474.

Radioiodination of DDA-TME conjugates
Fractions I and II were radioiodinated by the chloramine-T (Hunter & Greenwood 1962) and

Iodo-gen (Fraker & Speck 1978) methods. Both methods take less than 30 min and give acceptable products. For a chloramine-T radioiodination, 20 μg of Fraction I or II in methanol are mixed with 37MBq Na ^{125}I (Amersham International p.l.c., Amersham, Buckinghamshire, U.K.) and 20 μl of 80 μg ml^{-1} aqueous chloramine-T. After 30 s, 20 μl of 80 μg ml^{-1} aqueous sodium metabisulphite and 25 μl of 0·1 mg ml^{-1} aqueous potassium iodide are added. The reaction mixture is then transferred to a column containing about 1 ml Dowex-1 anion exchange resin (200–400 mesh) in the chloride form and eluted with 1 ml of water. The product is diluted to 10 ml with ethanol and stored in a silanized glass vial at −20 °C.

The Iodo-gen method is simpler than the chloramine-T method and so it was adopted for routine use. 5 μg of Fraction I or II in methanol is mixed with 18·5 MBq Na^{125}I (diluted to 25 μl with water) in a polypropylene microcentrifuge tube coated with 40 μg Iodo-gen (Pierce and Warriner (UK) Ltd, 44 Upper Northgate Street, Chester, Cheshire, U.K.). After 10 min with occasional mixing, the reaction mixture is diluted to 100 μl with water, purified on Dowex-1 resin and stored as described above.

Radioiodinated Fraction I was used routinely except where stated. A typical preparation is diluted (approximately 1:200) in buffer to give 10 000 counts min^{-1} per 50 μl.

Protocol: Assay A

The protocol is virtually identical to that used in previously-published methods (Rutterford & Smith 1980; Robinson et al 1980). Amounts of 50 μl each of sample or standard, [^{3}H](−)-methadone and antiserum are pipetted into duplicate sets of microcentrifuge tubes. Also, 50 μl [^{3}H](−)-methadone and 100 μl buffer are put in another pair of tubes to measure the total activity per tube. All tubes are vortexed, incubated at 2 °C for 1 h and then 475 μl PEG solution at room temperature (20 °C) are added. The tubes are vortexed thoroughly and allowed to stand at room temperature for 5–10 min before centrifuging (2 min, 12 000*g*). Aliquots, 400 μl, of the supernatants are transferred to 5 ml polypropylene liquid scintillation counting tubes, 4 ml scintillant are added and the [^{3}H](−)-methadone is extracted into the scintillant by 10 min vigorous shaking (extraction efficiency 100%). The tubes are then counted for 10 min in a liquid scintillation counter after standing in darkness in the counter for 15–30 min to allow the phases to separate and any luminescence of the samples or tubes to decay to zero, and the results are plotted.

Protocol: Assay B

Amounts of 25 μl of sample or standard and 50 μl each of radioiodinated Fraction I and antiserum are pipetted into duplicate sets of microcentrifuge tubes. The tubes are vortexed, incubated at room temperature for 30 min and then 400 μl of PEG solution are added. The tubes are vortexed thoroughly and allowed to stand for 5–10 min before centrifuging (2 min, 12 000*g*). The supernatants are aspirated and the precipitates are counted in a γ-counter. Two extra tubes containing only 50 μl radioiodinated Fraction I are counted to measure the total activity per tube, and the results are plotted.

H.p.l.c. determination of methadone in urine (I. Jane, unpublished)

Urine, 1 ml, is mixed with 2 M ammonium hydroxide, 1 ml, and internal standard (100 μl, 10 ng μl^{-1} aqueous imipramine hydrochloride). The mixture is extracted twice with 2 ml portions of redistilled diethyl ether which are combined and evaporated to dryness. The residue is taken up in 200 μl eluant consisting of 0·01 M ammonium chlorate in h.p.l.c. grade methanol (Fisons, Loughborough, Leicestershire, U.K.) adjusted to pH 6·7 with 0·1% v/v of 0·1 M sodium hydroxide in h.p.l.c. grade methanol. A valve injector is used to transfer 20 μl extract on to a 125 × 4·9 mm column of 5 μm particle-diameter S5W Spherisorb silica (Phase Sep, Deeside Industrial Estate, Clwyd, U.K.). The eluant flow-rate is 2 ml min^{-1} and the separated components are detected electrochemically (White 1979) with a potential of 1·0 V applied to a glassy carbon electrode relative to a silver/silver chloride reference electrode. The system is calibrated with blank urine 'spiked' with appropriate concentrations of (±)-methadone, and peak-height ratios of methadone to internal standard are plotted against concentration.

RESULTS AND DISCUSSION

The two methadone assays described are simple and economical.† Assay A has the advantage that the label as well as the antiserum is commercially available, but sample extraction and liquid scintilla-

† The running costs (excluding labour) are 6·5p per tube for Assay A and 3p per tube for Assay B.

tion counting are necessary. Assay B is simpler in practice and more economical than Assay A, but radioiodination facilities are required to prepare the label.

The characteristics of the two assays are given in Table 1. Non-specific binding (about 5% in each assay) is low enough to be ignored. Both assays are sensitive enough to detect methadone in blood or urine after therapeutic or illicit use. The sensitivity of Assay A is limited by the specific activity of the [^{3}H](−)-methadone, but that of Assay B may be varied over wide limits by preparing radioiodinated DDA-TME conjugates of different specific activities. The experimental details described in the previous section give a product with a relatively low specific activity (approximately 1 TBq mmol^{-1}; calculated from the activity in the product assuming 100% recovery) and this results in a standard curve that extends over a wide concentration range. Reducing the amount of DDA-TME conjugate in the radioiodination procedure gives a product with a higher specific activity. For example, radioiodination of 50 ng DDA-TME instead of 5 μg gives a product with a specific activity of approximately 30 TBq mmol^{-1}, i.e. ^{125}I is incorporated into about 40% of the DDA-TME molecules. When this radiolabel is diluted 1:50 to give about 10 000 counts min^{-1} per 50 μl and used with antiserum diluted 1:1500 (which binds 57% of the label), the standard curve extends over a useful concentration range of 0–2·5 ng ml^{-1} with 50% displacement of the label given by 1·0 ng ml^{-1} methadone. Such a sensitive assay could detect, for instance, 200 ng ml^{-1} or less of methadone in a portion of a bloodstain containing 5 μl of blood but, for routine blood or urine analysis, a less-sensitive assay with a wider concentration range is preferable.

Table 1. Characteristics of methadone assays.

	Assay A	Assay B*
'Cut-off'** (ng ml^{-1}):		
Blood	30 (n = 10)	33 (n = 54)
Urine	24 (n = 10)	21 (n = 100)
Coefficient of variation:***		
Intra-assay	10% (n = 10)	6% (n = 20)
Inter-assay	14% (n = 16)	11% (n = 15)
Mean recovery from 'spiked' blood***	85% (n = 10)	90% (n = 15)

* Using radioiodinated Fraction 1 as the label.
** Mean response of blank samples plus three standard deviations. Samples were obtained from healthy subjects who had not ingested methadone. Blood samples were haemolysed and varied in condition from fresh to putrid. Urine samples varied in age from several days to several months. Heparin, ethylenediaminetetraacetic acid (dipotassium salt), potassium oxalate or sodium fluoride present in some of the samples had no significant effect on the results.
*** Determined by assaying blood 'spiked' with 100 ng ml^{-1} (±)-methadone.

Assays A and B are virtually specific for methadone, the only other compounds with significant cross-reactivity being normethadone and α-methadol (Table 2). Methadone metabolites, dextropropoxyphene, dipipanone and phenadoxone have negligible cross-reactivities in both assays.

Table 2. Cross-reactivity data for methadone assays.

	Cross-reactivity*	
	Assay A	Assay B
Methadone	100	100
Normethadone	15	35
α-Methadol	10	15
Aminopentamide	—	5
Methadone-*N*-oxide	—	3
Isomethadone	—	1
(±)-Acetylmethadol	—	0·1

* Weight of methadone required to displace 50% of label × 100/weight of compound required to displace 50% of label.

The use of isomers rather than racemic mixtures as labels made it advisable to check whether the assays exhibited stereospecific effects. Standard curves of (+)-, (−)- and (±)-methadone were run on Assay A, and on Assay B with radioiodinated Fractions 1 and 2 as labels. The differences between the curves on each assay were minor and can be ignored in practice since 'spiked' samples give results close to those expected (Table 1) and, when 19 urines from addicts were compared by RIA (Assay B) and h.p.l.c., a correlation coefficient of 0·94 was obtained. Methadone concentrations in the urines ranged from 0·25–23 μg ml^{-1}. Assays A and B have been used in forensic case-work for 14 months and 8 months respectively and no problems have arisen.

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