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Determination of selected neurotransmitter metabolites using monolithic column chromatography coupled with chemiluminescence detection

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Abstract

The oxidation of selected clinically important neurotransmitter metabolites with acidic potassium permanganate in the presence of polyphosphates evokes chemiluminescence of sufficient intensity to enable the sensitive determination of these species. Limits of detection for 5-hydroxyindole-3-acetic acid (5-HIAA), vanilmandelic acid (VMA; α,4-dihydroxy-3-methoxybenzeneacetic acid), 4-hydroxy-3-methoxyphenylglycol (MHPG), homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid) and 3,4-dihydroxyphenylacetic acid (DOPAC) were between 5×10^{-9} and 4×10^{-8} M, using flow-injection analysis methodology. In addition, we demonstrate the rapid determination of homovanillic acid and 5-hydroxyindole-3-acetic acid in human urine – without the need for extraction procedures – using monolithic column chromatography with chemiluminescence detection.

Keywords: Neurotransmitter metabolites; Monolithic column chromatography; Chemiluminescence detection

1. Introduction

Abnormal production or release of hormonal or neuronal transmitters – serotonin, epinephrine, norepinephrine, dopamine (Fig. 1) – has been found to occur, or has been implicated, in numerous conditions including neurochromaffin tumours, orthostatic hypotension and psychiatric disorders. Therefore, the measurement of their metabolites – such as 5-hydroxyindole-3-acetic acid (5-HIAA), vanilmandelic acid (VMA; α,4-dihydroxy-3-methoxybenzeneacetic acid), 4-hydroxy-3-methoxyphenylglycol (MHPG), homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid) and 3,4-dihydroxyphenylacetic acid (DOPAC) – may assist diagnosis and subsequent patient monitoring [1]. Perhaps the most well-known example is the determination of HVA and/or VMA in urine to screen infants for the early stages of neuroblastoma [2], the most common extracranial solid tumour in childhood. Screening for neuroblastoma has been trialled in numerous countries including Canada, Germany and the USA. In Japan, mass screening was adopted as a national policy in the mid-1980s and continued until March 2004, when it was halted due to concerns about over-diagnosis and the effectiveness of the program [3]. A variety of analytical methodology has been used in these screening programs, including HPLC, ELISA and GC–MS [1,2,4]. These methods are time-consuming, can be prohibitively expensive or provide very limited information on the patient's biochemical profile.

Although a plethora of research on the determination of the parent neurotransmitters based on chemiluminescence detection has emerged (for example see Refs. [5–12]), there are surprisingly few reports of chemiluminescence determinations of their major urinary metabolites (Table 1). We have found that the oxidation of some clinically important neurotransmitter metabolites with acidic potassium permanganate in the presence of polyphosphates is accompanied by chemiluminescence of sufficient intensity for the sensitive determination of these species.

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Table 1
Chemiluminescence reactions used for the detection of selected neurotransmitter metabolites

Chemiluminescence reagents	Detection limits					References
	HVA	DOPAC	5-HIAA	VMA	MHPG	
Pre-column derivatisations						
6-Aminomethylphthalhydrazide		3×10^{-8} M	1×10^{-11} M			[21–23]
4-Dimethylaminobenzylamine (post-column peroxyoxalate chemiluminescence)			1×10^{-8} M			[24,25]
On-line reactions						
Luminol and hexacyanoferrate(III)	2×10^{-7} M			2×10^{-8} M		[16,26]
Sodium molybdate and hydrogen peroxide			Not stated			[27]
Manganese(IV)			Not stated			[28]
Permanganate			1×10^{-8} M			[18]
This study – permanganate						
Flow-injection analysis	3×10^{-8} M	2×10^{-8} M	5×10^{-9} M	4×10^{-8} M	1×10^{-8} M	
Monolithic column chromatography	1×10^{-7} M		2×10^{-8} M			

Compared to conventional HPLC, the use of monolithic columns allows high flow rates to be applied without a significant decrease in efficiency [13,14]. In addition to the decrease in analysis time, the higher flow rates improve compatibility between chromatographic separations and chemiluminescence detectors designed for flow-injection analysis [15]. In this paper, we present our investigations into the chemiluminescent oxidation of urinary neurotransmitter metabolites, and the application of this chemistry to the rapid determination of homovanillic acid and 5-hydroxyindole-3-acetic acid in human urine, using monolithic column chromatography with chemiluminescence detection.

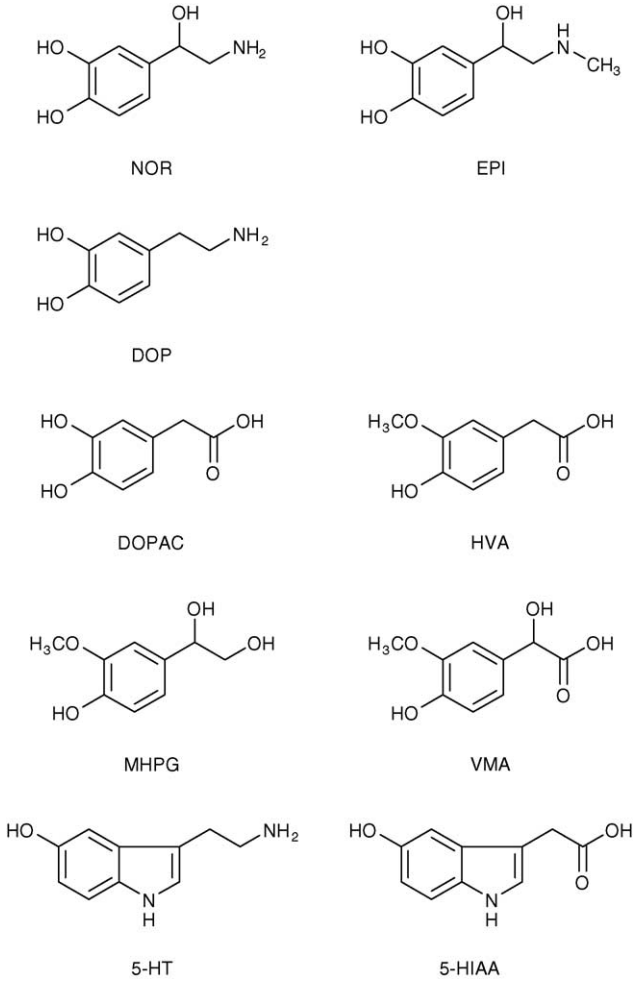

Fig. 1. Structures of norepinephrine (NOR), epinephrine (EPI), dopamine (DOP), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 4-hydroxy-3-methoxyphenylglycol (MHPG), vanilmandelic acid (VMA), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA).

2. Experimental

2.1. Instrumentation and procedure

The flow-injection analysis manifold (Fig. 2) consisted of a peristaltic pump (Gilson Minipuls 3, John Morris Scientific, Australia), bridged PVC tubing (1 mm i.d., PRO-TECH Group, Australia), PTFE tubing (0.8 mm i.d., Chromalytic Technologies, Australia) and six-port injection valve (Valco, SGE, Australia; 100 μL sample loop). Samples were injected into a water carrier stream that merged with the acidic potassium permanganate reagent stream at a T-piece (Embell Scientific, Australia), positioned 20 mm from a coiled PTFE flow cell. The flow cell was mounted flush against the window of a photomultiplier tube (PMT, Thorn-EMI, Model 9828SB, ETP Ltd., Australia), which was operated at 900 V provided by a stable power supply (Electron Tubes, Model PM20D) via a voltage divider (Thorn-EMI, Model C611, ETP Ltd.). The flow cell, photomultiplier tube and voltage divider were all enclosed in a light tight housing. The output from the photomultiplier tube was converted by a transimpedance am-

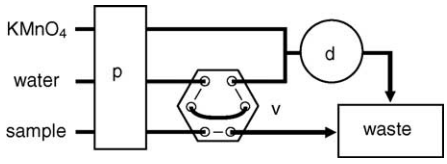

Fig. 2. Flow-injection analysis manifold, including (p) peristaltic pump, (d) chemiluminescence detector and (v) 6-port injection valve.

plifier (Thorn-EMI, Model A1, ETP Ltd.) and documented with a chart recorder (YEW Type 3066, Yokogawa Hokushin Electric, Japan). The Pulsed Flow Chemiluminescence Analyser (Precision Devices, Australia) used for the stopped-flow experiments has been described in detail in a previous publication [16].

Chromatographic runs were performed using a Hewlett Packard 1100 LC system that consisted of a quaternary pump, solvent degasser system and autosampler (Agilent Technologies). Sample components were separated with a monolithic column (Chromolith^{TM} SpeedROD RP-18e, 50 × 4.6 mm i.d.) and a solvent composition of 3% methanol in an aqueous solution of trifluoroacetic acid (0.1% v/v, pH 2) for two minutes followed by a linear increase in methanol concentration to 80% over the next eight minutes. Mobile phases were filtered through a 0.45 μm membrane. A flow rate of 3 mL/min and an injection volume of 20 μL were used for all experiments. For chemiluminescence measurements, the column eluate and potassium permanganate reagent were merged in the flow-through detector constructed for the flow-injection analysis manifold described above. A peristaltic pump was used to deliver the reagent. Data was acquired with Hewlett Packard Chemstation software.

2.2. Reagents

Deionised water (Millipore, MilliQ Water System, USA) and analytical grade reagents were used, unless otherwise stated. Solutions were prepared on a daily basis and pH measurements were made using a Jenko pH meter (CHK Engineering, Australia). Potassium permanganate (May and Baker, England) was prepared in a sodium polyphosphate (Aldrich, Australia) solution. The pH was adjusted with orthophosphoric acid (Ajax, Australia) as required. To prevent photo-degradation, the reagent flask was covered in aluminium foil. Serotonin, epinephrine, 5-HIAA, HVA, DOPAC, VMA and MHPG (Sigma, Australia) stock solutions (1×10^{-3} M) were prepared in deionised water.

3. Results and discussion

3.1. Investigations using flow-injection analysis

The chemiluminescence reagent, acidic potassium permanganate, has been used for the sensitive detection of a wide range of compounds [17], which includes biogenic amines; serotonin, epinephrine, norepinephrine and dopamine [6,18]. The urinary metabolites of these clinically important species have similar chemical structure to their parent compounds (Fig. 1), but only 5-HIAA has been determined with acidic potassium permanganate [18]. The emission arising from the oxidation of a variety of metabolites – 5-HIAA, HVA, DOPAC, VMA and MHPG – was examined and compared to that of serotonin and epinephrine, using flow-injection analysis with a chemiluminescence detector (Fig. 2).

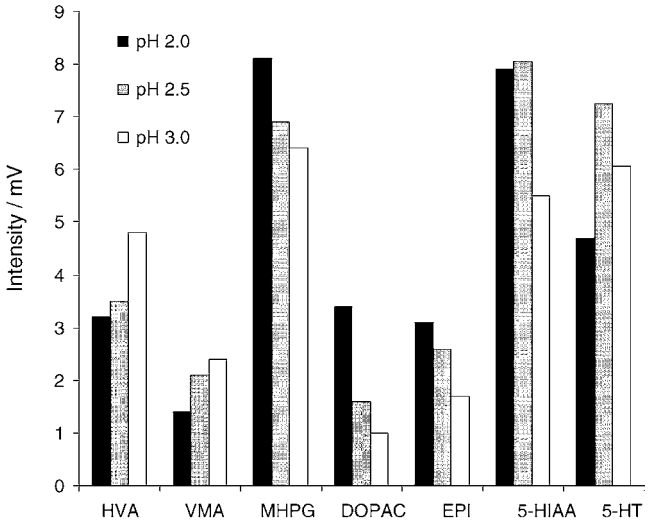

Fig. 3. Effect of pH on chemiluminescence intensity of 1×10^{-6} M VMA, HVA, MHPG, DOPAC, epinephrine (EPI), 5-HIAA and serotonin (5-HT). Each experiment was conducted with 7.5×10^{-4} M potassium permanganate and 1% sodium polyphosphate. The intensities for 5-HIAA and 5-HT have been divided by 2 and 20, respectively, for comparison purposes.

The chemiluminescence intensity was dependent on reagent pH, and although the metabolites have similar chemical structures, the optimum pH was not consistent (Fig. 3). The flow rate was optimised for each analyte under each of the reagent conditions. The chemiluminescence intensities for the two indoles, 5-HIAA and serotonin, were greater than the other compounds, which resulted in lower limits of detection for 5-HIAA (5×10^{-9} M) and serotonin (7×10^{-10} M), compared to the other species; HVA, DOPAC, VMA, MHPG and epinephrine (between 1×10^{-8} and 4×10^{-8} M).

3.2. Stopped-flow examination of reaction kinetics

During investigations with flow-injection analysis it was noted that the optimum flow rate for the indoles (5-HIAA and serotonin) was greater than that for the other analytes. This was explored further by examining the intensity versus time profile of the chemiluminescence accompanying the oxidation of VMA, HVA, 5-HIAA and serotonin, using the stopped-flow mode of a Pulsed Flow Chemiluminescence Analyser [16]. The temporal distribution of the chemiluminescence emanating from a reaction intermediate in static solution is largely dependent on the kinetics of the chemical reaction [19]. The light-producing pathways for the oxidation of the two indoles progress relatively quickly; the emission accompanying both reactions neared completion in one second. In contrast, the chemiluminescence from the oxidation of VMA and HVA persisted for over 10 s, although the maximum intensity occurred at approximately 0.5 s. Typical profiles for the oxidation of 5-HIAA and HVA with acidic potassium permanganate are shown in Fig. 4.

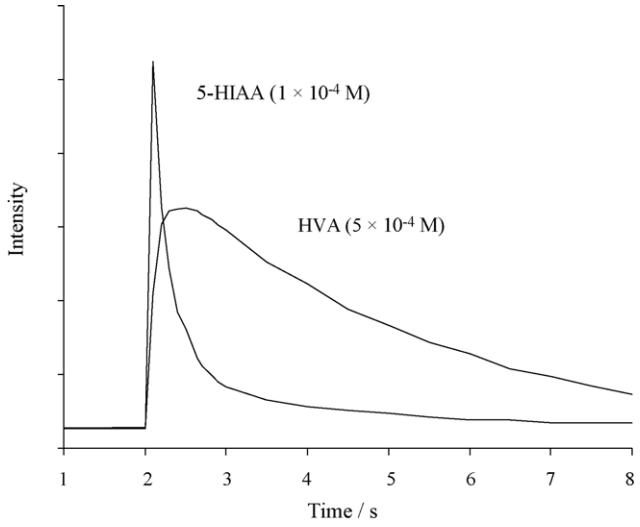

Fig. 4. Intensity vs. time profiles (smoothed to remove noise) produced with the stopped-flow mode of a Pulsed Flow Chemiluminescence Analyser.

3.3. Monolithic column HPLC with chemiluminescence detection

The chemiluminescent oxidation of neurotransmitter metabolites was applied to the rapid determination of HVA and 5-HIAA in human urine, by coupling the chemiluminescence detector (flow-cell and photomultiplier tube) to the exit line of a HPLC system. The use of a monolithic column allowed flow rates that were comparable to those used in flow-injection analysis, and separation of the two analytes from all interferences in less than five minutes (Fig. 5). The samples were filtered, but no extraction procedure was required. The retention times for HVA and 5-HIAA were 3.45 and 4.10 min (<3% RSD). The analysis of urine that had been spiked with neurotransmitter metabolites revealed that the dopamine metabolite DOPAC could also be resolved (retention time of 1.76 min), but we did not detect significant amounts of this species in the urine samples (provided by healthy volunteers). Under the current separation conditions, MHPG and VMA (retention times of 0.82 and 0.31 min) were partially or completely obscured by other (yet to be characterised) species in urine that evoke chemiluminescence on reaction with acidic potassium permanganate. A comparable signal was also obtained for serotonin (retention time of 1.08 min), due to the fortuitous combination of the greater emission intensity for this analyte (compared to the neurotransmitter metabolites) using chemiluminescence detection, and its significantly lower concentration in the urine samples (normal range of 0.3–1.3 μmol/day compared to 10–42 μmol/day for 5-HIAA [20]). As shown in Table 1, the detection limits for HVA and 5-HIAA in urine were close to that observed using flow-injection, due to the flow-rate compatibility of the separation and detection. The minor decrease in sensitivity could be attributed to the smaller injection volume, greater broadening of the sample zone and the compromises made in the reagent conditions for the optimum detection of both analytes.

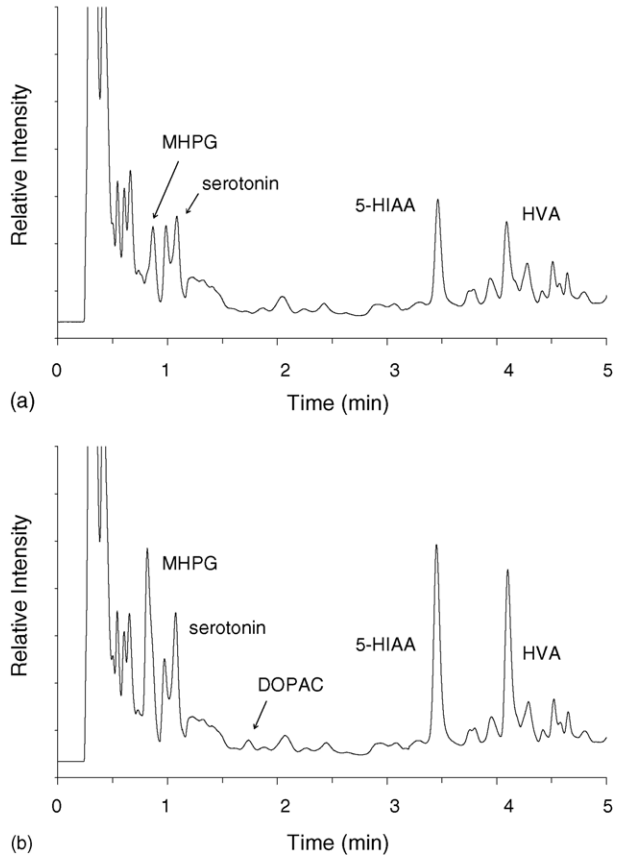

Fig. 5. Typical chromatogram for the determination of HVA and 5-HIAA in (a) urine from a normal human subject and (b) the same urine spiked with 5×10^{-5} M HVA, DOPAC, VMA and MHPG, 3×10^{-5} M 5-HIAA, and 1×10^{-6} M serotonin, using monolithic column separation and chemiluminescence detection (7.5×10^{-4} M potassium permanganate in a 1% sodium polyphosphate solution adjusted to pH 2.5 with orthophosphoric acid).

3.4. Comparison with other chemiluminescence methods of detection

Previously reported chemiluminescence-based methods for the determination of neurotransmitter metabolites that have been applied to chromatographic separations each involved off-line (pre-column) derivatisation and subsequent post-column reaction of the derivatised chemiluminophores [21–25] (Table 1). Although these methods are quite sensitive and could possibly be extended to include other metabolites of interest, the derivatisation and time-consuming separation (20–35 min [21,22]) are not ideal for mass screening applications. In one study, exceptional detection limits for 5-HIAA of 1×10^{-11} M were reported, but in real samples an overwhelming response from the preceding peak reduced the effective detection limit to 2×10^{-7} M.

The other chemiluminescence-based methods [16,18,26–28], including the only previous reports of

chemiluminescence detection of VMA [26] and HVA [16], are exploratory flow analysis studies involving the combination of a chemiluminescence reagent with single analyte standards. The study presented in this paper is the first use of a post-column on-line chemiluminescence reaction (without prior derivatisation) for the determination of neurotransmitter metabolites. With the exception noted above, the sensitivity of this procedure is comparable with all previously reported chemiluminescence-based determinations and suitable for the determination of these analytes in clinical samples [1]. The selectivity of chemiluminescence detection (using reagents such as acidic potassium permanganate) reduces the number of interferents that must be separated and therefore enhances the advantages of the monolithic column approach.

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